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HISTOCHEMICAL STUDIES ON THE CEREBRAL LIPIDOSES AND OTHER CELLULAR METABOLIC DISORDERS *

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A number of diseases of widely varying clinical behavior have the common property of producing swollen and microscopically homogeneous or hyalinized nerve cells. Some have been grouped as the amaurotic idiocies, but others have been considered generally to be unrelated to this group. Recent advances in chemical knowledge of the lipids in affected ganglion cells in certain of these conditions, and of the various lipid or polysaccharide substances found in the visceral cells of others, suggested that a comparative histochemical study would be of value. In addition to clarifying the relations of these various diseases to each other, it was hoped that such a study might provide means by which they could be differentiated in pathologic practice. It was felt also that additional light might be shed on the histochemical significance of some of the lipid-staining procedures now in general use.

A classification of the various entities of interest in this group of diseases, together with present knowledge of the nature of the lipids or other substances which accumulate in the affected cells, is presented in Table I. Formalin-fixed tissue, obtained from representative examples of most of these conditions, was utilized in this study, either as frozen sections or as sections of tissue dehydrated in ethanol and xylol. The histochemical and conventional staining procedures utilized will be discussed. Frozen and paraffin sections of nine normal brains served as control material.

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TABLE I

Classification of Metabolic Disorders Affecting Ganglion Cells or Producing Storage of Lipids in Viscera

Conditions	References	Substance in		Material studied		
		Ganglion cells	Visceral cells	No. of cases	Tissue available	Preparations (all tissue fixed in formalin)
A. Neural lipid disorders or amaurotic idiocies (cerebral, cerebrospinal, cerebroretinal, or cerebromacular lipidoses)	1–6, 13, 27					
1. Infantile form: Tay-Sachs disease		Ganglioside	None	1	Brain	Paraffin
2. Early juvenile form: Bielschowsky-Jansky disease		?	None	1	Brain	Frozen and paraffin
3. Late juvenile form: Spielmeyer-Vogt disease		?	None	1	Brain	Paraffin
4. Adult form: Kufs's disease		?	None			
B. Neurovisceral metabolic disorders						
5. Niemann-Pick disease	1, 26, 27	Ganglioside	Sphingomyelin	2	Liver and spleen	Frozen and paraffin
				1	Brain	Paraffin
6. Periarticular Niemann-Pick-like disease	28	?	Sphingomyelin?			
7. Gargoylism (Hurler or Hurler-Pfaundler disease)	9–12, 29	?	Mucoprotein and lipoprotein	1	Brain	Paraffin
8. Infantile Gaucher's disease ?*	1, 31	None?	Cerebroside			
C. Visceral lipid disorders						
9. Adult Gaucher's disease	30, 32–34	None	Cerebroside	1	Liver and spleen	Frozen and paraffin
10. Adult Niemann-Pick-like disease	35	?	Sphingomyelin, cerebroside	1	Spleen	Frozen and paraffin
11. Letterer-Siwe disease†	1	None	Neutral fat and cholesterol	1	Skin	Frozen and paraffin
D. Infantile progressive muscular atrophy						
12. Werdnig-Hoffmann disease	7, 8	?	None	3	Brain	Frozen and paraffin

* Patients with the acute infantile form of Gaucher's disease do have cerebral symptoms, but there is apparently no evidence that this is the result of lipid accumulation in nerve cells.[1]

† Letterer-Siwe disease is included in this table because it is commonly considered a metabolic disorder; however, this disease, and its allies (eosinophilic granuloma, Hand-Schüller-Christian disease) are probably acquired diseases rather than genetically determined metabolic disorders.

Results

General

Abnormal ganglion cells in all the conditions studied were enlarged or rounded, and stained weakly with such survey stains as hematoxylin and eosin. Their morphologic features have been described in detail by many writers.[1-18] Affected nerve cells in groups A and B of Table I tended to show preservation of perinuclear cytoplasm at the axonal pole and replacement of the dendritic end by a pale homogeneous or finely granular mass, the staining properties of which will be described. This polarity was least well shown in Tay-Sachs disease. Involvement of axons was seen in all, but was perhaps most striking in Hurler's disease. Ganglion cells of the spinal cord, and similar cells outside the central nervous system (as in sympathetic ganglia and the myenteric plexuses), also were affected in these several conditions. Cells at the medullary and higher levels in Tay-Sachs disease tended to be more swollen than such cells in the other conditions, while comparable cells in Spielmeyer-Vogt disease were usually more hyaline in appearance. Attempts to identify unlabelled slides, however, have shown that these conditions could not be distinguished reliably by examination of sections stained by routine survey procedures.

In Werdnig-Hoffmann disease, on the other hand, only scattered ganglion cells were swollen and hyalinized, the other cells appearing normal. The spinal cord and brain stem, at least to the thalamic level, were involved, but in none of the three cases studied were affected cerebral cells found. The distribution of affected cells, their rarity in sections (apparently due to the fact that they lyse and disappear with time), the normal appearance of surviving ganglion cells, and the lack of involvement of ganglion cells outside the central nervous system and the spinal nerve ganglia all aided in the microscopic distinction of this condition from the others studied.

Study of paraffin sections of formalin-fixed material, and of frozen sections in those few instances in which this was possible (Table I), showed that the affected ganglion cells in all the neural and neurovisceral lipid disorders contain lipids which are stainable with Sudan dyes even after embedding. The ganglion cells of Werdnig-Hoffmann disease are Sudan-negative after embedding. Of the abnormal visceral cells studied, only those of Niemann-Pick disease and the adult Niemann-Pick-like disease stain strongly with Sudan dyes after embedding, the cells of Letterer-Siwe, Gaucher's, and Hurler's diseases being Sudan negative after such treatment.

Many normal nerve cells in the nine control brains showed a small, round, or irregular mass of lipid, usually in the axonal pole near the

nucleus. Usually this was most prominent in the cells of the inferior olive; it often resisted extraction and could be stained in sections of embedded tissue, but it was more soluble than the abnormal cytoplasmic lipids under discussion. Detailed studies of this structure were not carried out, but its site suggests that it may be the Golgi apparatus, the lipoprotein nature of which is well known.[14]

Histochemical Data

In Table II are presented the results obtained by staining paraffin sections of formalin-fixed brains by a number of procedures of histochemical interest. As previously noted, lipid stainable by Sudan dyes was present after paraffin embedding in all ganglion cells studied except those of infantile progressive muscular atrophy. The greatly swollen cells of Tay-Sachs disease react rather weakly, but the intensity of staining is approximately the same in all the others. Only the cells of Tay-Sachs disease failed to give a peracetic or performic acid-Schiff reaction, procedures which demonstrate, among other substances, unsaturated lipids.[15-17] The phosphomolybdic acid-stannous chloride (PMA) procedure,[18] positive in all ganglion cells studied, apparently has no value in differential diagnosis, nor can it be considered specific for lipids of any type. The significance of the positive phosphotungstic acid-hematoxylin stain in the affected cells in Niemann-Pick and Hurler's diseases is unknown.

With the exception of those in Werdnig-Hoffmann disease, all ganglion cells studied stained with certain basic dyes, including thionine, crystal violet, iodine green, Nile blue, and neutral red, but not with others, including cresyl violet, basic fuchsin, methyl green, methylene blue, and safranine. All acid dyes failed to stain.

Among visceral cells, only Niemann-Pick cells, and those in the adult visceral Niemann-Pick-like disorder, retain significant staining power after embedding. Sphingomyelin, the lipid present in visceral cells in Niemann-Pick disease, thus shares in some respects the behavioral properties of the ganglion cell lipids just described, whereas cerebroside (in Gaucher's disease), and neutral fat and cholesterol (in Letterer-Siwe disease), do not. It is apparent also that the PMA procedure does not distinguish sphingomyelin, which contains choline, from ganglioside, which does not. The significance of the positive periodic acid leukofuchsin reaction of the lipids known to be present in the conditions listed in Table II is not always clear. Ganglioside (brain, Tay-Sachs) and cerebroside (viscera, Gaucher's) do contain carbohydrate residues, but sphingomyelin does not, nor does it contain a free glycol or analogous group to give the reaction. Whether the data

TABLE II

Staining Properties of Ganglion and Visceral Cells in Paraffin Sections

	Sudan IV	PMA	PAS	PTAH	PFS	Iodine green	Direct Schiff	Direct aldehyde-fuchsin	Sulfation aldehyde-fuchsin	Phosphorylation aldehyde-fuchsin
Ganglion cells										
Normal	−*	+	±	−	±	Purple	−±	−	±	2+
Tay-Sachs	±	±	± to −	−	−	Purple	−	−	+	+
Bielschowsky-Jansky	+	+	+	−	+	Purple	−	+	+	3+
Spielmeyer-Vogt	+	+	3+	−	2+	Purple	±	−	2+	4+
Niemann-Pick	+	+	+ to ±	+	2+	Purple	±	−	2+	+
Hurler's	+	+	2+	±	+	Purple	±	±	2+	2+
Werdnig-Hoffmann	−	+	±	−	±	Pale violet	±	−	+	2+
Visceral cells										
	Sudan dyes									
Niemann-Pick	+	+	2+	−	+		±	−	+	3+
Gaucher's	−	−	±	−	−		−	−	±	2–3+
Letterer-Siwe	−	−	−	−	−		−	−	±	±
Adult Niemann-Pick-like disease	+	+	+							

PMA = Phosphomolybdic acid-stannous chloride stain; PAS = periodic acid-Schiff stain; PTAH = phosphotungstic acid hematoxylin stain; PFS = Performic acid-Schiff stain.

* With the exception of the juxtanuclear droplet, presumably Golgi apparatus, as is discussed in the text.

indicate that ganglioside, cerebroside, or other potentially reactive substances always occur in association with sphingomyelin cannot be stated, but evidence that this interpretation may be correct will be presented.

As is shown in Table II, all abnormal cells studied, except possibly those of Letterer-Siwe disease, contain entities which can be converted to acidic groups by sulfation[19] or phosphorylation.[20] Whether the reactive groups concerned are those of carbohydrate moieties of lipids, or aliphatic hydroxyl (serine, threonine), groups of proteins cannot be stated. However, these data are consistent with the interpretation that carbohydrate residues, adequate in number to account for the periodic acid-Schiff (PAS) reactions observed, are always present as components of, or in association with, the lipoproteins under discussion.

In an effort to determine whether more than one stainable entity is present in abnormal ganglion cells of the type under study, paraffin sections from patients with Bielschowsky-Jansky disease, Niemann-Pick disease, and the adult visceral Niemann-Pick-like disease were treated by a variety of solvents and reagents, and then stained by the PMA, periodic acid leukofuchsin, and Sudan procedures. The PAS positivity of nerve cells of Bielschowsky-Jansky disease was significantly decreased by pyridine at 57° C. after 4 days' exposure, and by ethanol at 57° C. after 6 days, whereas ethanol and pyridine at 27° C., and acetone and chloroform at 57° C. had no significant effect over 6 days. All of these solvents except chloroform at 57° C. produced some decrease in Sudan IV staining of splenic Niemann-Pick cells, but no reduction of the PAS reaction of these cells was obtained with any of these solvents, even after extraction for 6 days at 57° C. Other solvents found capable of reducing sudanophilia of Bielschowsky-Jansky cells include water at 100° C., xylol-acetic acid (ratio, 30/70 parts) and 30 per cent hydrogen peroxide. Solvents found to be incapable of producing this effect include methanol, ethyl ether, and xylol. Some decrease in the PMA staining power of the lipid in these cells was obtained with 1 N hydrochloric acid, 1 N sodium hydroxide, 1 per cent barium hydroxide, ethanol, pyridine, boiling water, xylol-acetic acid and acetone-ether (equal parts). No effect on the PMA stain was obtained with aqueous solutions of oxalic acid, potassium dichromate, ferric chloride, iodine-potassium iodide, or lead nitrate. These data are not presented in detail because they are not considered to shed light, at present, on the finer chemical structure of the lipids of these cells. The apparent dissociation of the Sudan and PAS staining powers of Niemann-Pick cells is, however, consistent with the interpretation that a carbohydrate-containing entity is present in association with sphingomyelin in that

disease. Craig,[21] in analyzing the behavior of Hale's colloidal iron stain on sections treated with organic solvents, has reached similar conclusions.

The greater solubility of these lipids in more polar than in less polar* solvents suggests that they are present in large part, if not entirely, as lipoproteins rather than as pure lipids or as proteolipids. The coupled tetrazonium[22] and Hartig-Zacharias ferrocyanide[23] reactions for protein are positive in the lipid areas of affected Bielschowsky-Jansky cells, although the Millon[24] and xanthoproteic reactions are negative. Myelin, which, as was mentioned, gives staining reactions much like those of the abnormal cells studied, was found to be much more soluble in many of these organic extracting agents, indicating that the lipids of myelin occur in higher proportion relative to protein (proteolipids?), or are much less firmly bound to protein.

In order to analyze more fully the significance of the various staining reactions described, a variety of relevant lipids commercially obtainable were prepared in three different ways, and stained by the procedures given in Table II. The lipids were (a) mixed with Mayer's glycerine-albumin and drops dried on slides; (b) mixed with gelatin solution and drops dried on slides; (c) dried from aqueous or organic solutions on glass-fiber filter paper. The results of these studies are presented in Table III, which shows clearly that the staining of the various lipids depends, at least in part, on the method of preparation of the lipid for staining. In general, stronger stains were obtained with the preparations dried on glass-fiber filter paper, but many unexplainable discrepancies exist. On filter paper, lecithin, cephalin, and sphingomyelin all gave some stain by most procedures which stain the lipid storage cells under discussion, whereas in gelatin, cephalin showed the greatest similarity to the staining properties of the cells. On the other hand, in albumin-glycerol, sphingomyelin resembled most strongly the cellular lipids. These data may indicate that sphingolipids are best able to account for the staining properties of affected brain cells in the various neural and neurovisceral lipid disorders studied, but are certainly not adequate to prove that only sphingolipids can do so.

Discussion

Chemical data available at present indicate that affected ganglion cells in Niemann-Pick and Tay-Sach diseases contain excess ganglioside whereas visceral cells in Niemann-Pick disease contain sphingomyelin.[1]

* Polar is used with reference to ability to dissolve polarized molecules. For example, xylol and chloroform are less polar than ethanol, methanol, and acetone, which, in turn, are less polar than water.

TABLE III

In Vitro Staining Reactions of Lipids

	Sudan black	Direct Schiff	PAS	PCS	PMA	PTAH	Smith-Dietrich	Crystal violet
(1) Dried on glass-fiber filter paper								
Crude lecithin*	2+	2+	3+	2+	+	+	+	2
Lecithin	2+	+	3+	2+	o	+	+	2
Cephalin	2+	2+	2+	2+	2+	o	2+	3+†
Sphingomyelin	o	±	2+	o	o	+	+	±
Sphingosine	o	o	+	o	o	o	+	o
Kerasin	±	+	+	+	o	o	+	2+
Crude myelin‡	o	±	+	o	+	o	2+	2+
Triacetin	o	o	o	±	o	o	o	o
Tristearin	2+	o	±	o	o	±	±	o
(2) In gelatin								
Crude lecithin*	o	+	o		o		+	+
Lecithin	2+	o	2+		o		o	2+
Cephalin	2+	+	3+		±		o	3+
Sphingomyelin	+	o	3+		+		±	±
Sphingosine	+	o	2+		+		±	+
Kerasin	o	±	3+		o		o	+
Gelatin control	o	o	±		o		o	+
				PFS				
(3) In albumin-glycerol								
Crude lecithin*	o	o	o	o	o	±	o	o
Lecithin	o	o	o	o	o	±	o	o
Cephalin	±	±	o	o	±	±	o	±
Sphingomyelin	2+	+	o	o	2+	+	2+	2+†
Sphingosine	o	o	o	o	o	±	o	o
Kerasin	o	o	o	o	±	±	o	o
Albumin-glycerol control	o	o	o	o	o	±	o	o

PAS = Periodic acid-Schiff stain; PCS = peracetic acid-Schiff stain; PMA = phosphomolybdic acid-stannous chloride stain; PTAH = phosphotungstic acid-hematoxylin stain; PFS = performic acid-Schiff stain.

* Crude lecithin is a mixture of lecithin and cephalin.

† Metachromatic staining reaction.

‡ A dried pyridine extract of normal brain.

The chemical nature of the lipoprotein in nerve cells in Hurler's disease and the juvenile and adult amaurotic idiocies is not known.[1,27,29] As is shown in Table II, affected ganglion cells in Werdnig-Hoffmann disease differ basically from those in the other conditions studied, in that they do not contain lipid in amounts detectable by the methods used. The resistance to solution of the lipid in affected nerve cells in the neural and neurovisceral lipidoses has been noted by many previous workers.[4,11,18,21,25] Although comparison of frozen and paraffin sections of the same tissue stained by the same methods has shown relatively little decrease in staining power (for Niemann-Pick disease and the several cerebral lipidoses), the possibility that the procedures of dehydration and embedding may have resulted in fractionation of an original lipid or lipoprotein mixture, with retention only of the least soluble components, cannot be denied. If this actually is the case, the conclusions drawn from the histochemical data can in general be applied only to this less soluble residuum.

The similarity of the staining and behavioral properties of normal myelin of the central nervous system to those of the lipids in the several types of abnormal cells under discussion, and to the paranuclear lipid droplet of normal nerve cells, is of considerable interest. The degree of significance that can be given to this similarity, and the possible relation of the Golgi apparatus to the presence of lipoprotein in increased amount in certain types of abnormal ganglion cells, cannot be stated at present.

As has been mentioned, the data on *in vitro* staining of lipids suggest that sphingolipids are best able to account for the staining properties of affected cells in the amaurotic idiocy complex, Niemann-Pick disease and its allies, and the brain cells of Hurler's disease. These data, however, also demonstrate that even *in vitro* tests on purified compounds may not clarify the analysis of lipid staining properties. They indicate, further, that considerable caution is necessary in drawing conclusions as to the chemical nature of intracellular lipids by a comparison of the staining behavior of sections and lipid preparations. In order to permit such conclusions, much greater precautions with regard to the purity of the lipid preparations used would be required than was possible in this study.

Histochemical differentiation of the cerebral lipidoses and the neurovisceral lipid metabolic disorders apparently hinges on the possibility of distinguishing ganglioside from sphingomyelin, and from other sphingolipids which may be present in the affected cells in those diseases as yet incompletely studied chemically. Although the data presented in this paper have shown no clear-cut differences in histo-

chemical properties of affected cells in various members of these two disease groups, advances in technique may permit such distinctions to be made in the future. The lipids of as yet unknown nature in affected nerve cells in Hurler's, Bielschowsky-Jansky, and Spielmeyer-Vogt diseases show the same general staining properties as those in Tay-Sachs and Niemann-Pick diseases; the results of the histochemical studies described do not, however, permit more definite identification of them.

At present then, the abnormal cellular components of affected visceral cells in Gaucher's, Letterer-Siwe, Hurler's, and Niemann-Pick diseases can be separated from each other histochemically, but the visceral Niemann-Pick-like disease of adults cannot be separated from the last. The abnormal nerve cells of Werdnig-Hoffmann disease can be separated from those of all other conditions studied, but the various lipid disorders of nerve cells cannot be clearly distinguished from each other histochemically.

Summary

The results of histochemical studies on affected cells of a number of neural and visceral lipid metabolic disorders (including the infantile and early and late juvenile forms of amaurotic idiocy, Niemann-Pick disease, and the adult Niemann-Pick-like disease, Gaucher's disease, and gargoylism) are presented and compared. Similar studies on Letterer-Siwe disease and Werdnig-Hoffmann's infantile muscular atrophy are also reported.

Affected ganglion cells in all conditions studied except Werdnig-Hoffmann disease appear to contain protein-bound lipid which is insoluble in organic solvents after formalin fixation. Affected visceral cells show this property in Niemann-Pick disease and in the adult Niemann-Pick-like disease. The various staining properties described are best explained by the occurrence of sphingolipids in all of these conditions, but the data do not permit decision as to how many different forms may be present, either in cells in the same disease, or in the group of diseases as a whole.

The utility of Sudan staining after paraffin embedding in the study and diagnosis of metabolic diseases characterized by storage of lipids of this type is stressed. It has not been possible to decide whether sphingomyelin and ganglioside can be distinguished by available histochemical procedures. The phosphomolybdic acid-stannous chloride procedure cannot be regarded as reliably indicating the site of lipid-bound choline or allied basic substance.

Data demonstrating the unreliability of *in vitro* tests on purified

lipids, in the analysis of the staining properties of intracellular lipoprotein complexes, are presented.

ADDENDUM

Since submission of this manuscript for publication, our attention has been drawn to the detailed study of Diezel (Diezel, P. B. Histochemische Untersuchungen an primären Lipoidosen: Amaurotische Idiotie, Gargoylismus, Niemann-Picksche Krankheit, Gauchersche Krankheit, mit besonderer Berücksichtigung des Zentralnervensystems. *Virchows Arch. f. path. Anat.*, 1954, **326**, 89–118). Diezel concluded that the abnormal ganglion cells in the conditions which we designated by numbers 1, 2, 3, 5, and 7 in our Table I contain both ganglioside and sphingomyelin, the proportion of the latter being lowest in Tay-Sachs disease and highest in Niemann-Pick disease. Although the histochemical findings of his study are in accord with those of the present paper, we consider that the methods available at present do not permit so definite a conclusion as to the number of molecular entities in the cells under discussion.

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MORPHOLOGIC AND FUNCTIONAL CHANGES IN THE LIVERS OF RATS AFTER LIGATION OR EXCISION OF THE COMMON BILE DUCT *

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Clinical studies with large doses of parenterally injected, buffered chlortetracycline have shown that the best therapeutic results on patients with inoperable cancer were obtained when the patient developed severe jaundice during therapy.[1,2] Since the relationship between liver function and tumor growth constitutes one of the central problems in experimental cancer research, these observations led to studies on the effects of non-specific liver injury on transplantable rat and mouse tumors.[3] In the course of that investigation it became apparent that more basic knowledge concerning the morphologic and functional changes during experimentally produced hepatic insufficiency was essential for understanding the effects observed. In this communication the results of a study of the functional and morphologic changes in the rat liver after excision and simple ligation of the common bile duct are reported.

MATERIALS AND METHODS

Three-months-old male Osborn-Mendel rats were divided into three groups: group I consisted of 25 rats in which the common bile duct was excised; group II consisted of 21 animals in which the common bile duct was ligated; and group III consisted of 23 rats which were observed as unoperated or sham-operated controls.

Surgical Procedures

Excision of the common bile duct (group I). Eight to 10 mm. of common duct were excised under ether anesthesia by the ventral approach. The structures contained in the hepatoduodenal ligament were dissected under a microscope. Special care was taken to avoid severance of the hepatic artery or the portal veins. The proximal and distal stumps of the remaining duct were tied off with three silk ligatures each.

Simple ligation of the common bile duct (group II). Ligation of the common

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duct was carried out by placing a figure eight loop around the midportion of the duct and securing it with two or three ties of fine surgical silk.

Sham operations (group III) were performed by opening the peritoneal cavity, visualizing the bile duct, and closing the excision 15 to 20 minutes later.

Animal Maintenance and Necropsy

Animals were operated upon in groups of 6 or 12 rats. They were then transferred to metabolism or standard stock cages and given food and water ad libitum. Urine from a number of rats was collected under toluene.

The rats were sacrificed by exsanguination (cardiac puncture) under nembutal anesthesia at *1, 3, 7, 14, and 28 days* after the operation. Experimental and control animals were sacrificed simultaneously. The exsanguinated blood was collected and stored for further studies. The weights of the body, the livers, the adrenal glands, and the testes were recorded at necropsy (Table I). Tissues were fixed in Zenker's

TABLE I

Data for the Three Groups of Experimental Animals. The Weights Given Are Averages for the Respective Sub-Groups

	Interval between operation and necropsy	Animal weight		Liver weight per 100 gm. of rat	Adrenal weight per 100 gm. of rat	Survived
		Operation	Necropsy			
	days	*gm.*	*gm.*	*gm.*	*mg.*	
Group I	1	313	312	4.03	18.7	3/3
	3	311	327	4.54	16.5	3/3
	7	197	192	6.09	22.5	3/3
	14	196	199	6.76	21.6	3/3
	28	253	232	9.52	25.6	2/7
Group II	1	266	263	3.55	11.4	4/4
	3	296	267	3.98	12.6	4/4
	7	211	198	6.07	13.7	4/4
	14	201	209	5.51	15.1	3/4
	28	159	198	4.34	14.7	5/5
Group III	0 (controls)		242	3.88	13.1	23/23

acetic acid fluid, Carnoy's fluid, and in 10 per cent formalin, and embedded in paraffin. Sections of liver were stained with hematoxylin and eosin and for alkaline phosphatase.

Laboratory Procedures

Total serum proteins, serum non-protein nitrogen, and urinary albumin were determined by the micro-Kjeldahl method (nesslerization).

Combined and free serum bilirubin were estimated by the van den Bergh method of diazotization.

Serum proteins were fractionated by paper electrophoresis. Quantitative measurements of the serum protein fractions were carried out by the dye-staining and elution method, using the colorimetric determination of brom thymol blue.[4]

Quantitative evaluation of the cytologic changes taking place in the liver was obtained by the method of Chalkley.[5] Two to four sections of liver from each animal were evaluated and 600 to 800 cytologic units scored per section.

Results

Group I

Marked yellow discoloration of the skin in rats with partial excision of the common bile duct was observed within 24 to 72 hours after the operation. Increasing weight loss was noted in rats which were observed for more than 7 days. After 1 week, anorexia was evident in these animals. Fourteen or more days after excision of the duct the rats became markedly lethargic and occasionally exhibited disturbances of equilibrium. Only 2 of 7 rats survived for 28 days following the operation, at which time they were moribund.

Gross Necropsy Findings

Twenty-four hours after excision of the duct, except for a slight dilation of the proximal stump, no remarkable gross changes had taken place. After *3 days,* dilation of the proximal stump was more marked and the liver appeared slightly enlarged. The edges of the liver had become slightly rounded and the whole organ appeared congested. Seven days after excision, the proximal stump was quite dilated, containing from 1.0 to 5.0 ml. of a yellowish white mucous fluid. The liver was markedly enlarged and its yellow discoloration was distinctive. The kidneys appeared swollen, soft, and pale. The subcutaneous tissues and omenta in these animals were bright yellow and slightly edematous. *Fourteen* (Fig. 1) to *28 days* after excision of the bile duct the dilation of the stump had progressed even more, sometimes containing as much as 20 ml. of a white mucoid fluid. The liver was notably enlarged and patches of bright yellow discoloration were apparent. The larger lobes generally were more affected than the smaller lobes. In some animals an accumulation of clear yellow ascitic fluid was observed. To test whether minute ruptures were present in the dilated stump, trypan blue was injected through a number 27 needle into the dilated duct. Since no dye passed from the dilated duct, it was assumed that the yellow ascitic fluid accumulated by transudation.

All of these animals showed enlargement of the adrenal glands, the color of which was gray-red. The kidneys had a distinct greenish color, appeared enlarged, and in some instances showed gray-white spots on the surface. Upon sectioning, in some of the kidneys dilation of the renal pelvis similar to that in hydronephrosis was found.

Edema of the subcutaneous tissues was notably increased as compared to that in the 7-day group. The subcutaneous and mesenteric fat was decreased.

Microscopic Findings

One day after the operation the protoplasm of the liver cells adjacent to the central veins stained weakly with hematoxylin and eosin, in sharp contrast to the periphery of the lobules which stained brightly (Fig. 2). The liver cells in the central portions also showed a less distinct delineation of the cellular membranes. Small necrotic areas were evident in all liver sections, with some of these areas showing infiltration by leukocytes.

Three days following excision of the duct the liver cells were vividly stained in all portions of the lobules. It was noted that more cells exhibited anisocytosis and anisokaryosis, and more liver cell nuclei contained more than one nucleolus. Liver cells in mitosis were frequent. The necrotic areas were present and in an early stage of absorption. The interlobular bile ducts were dilated and the portal septa were enlarged. A large number of newly formed bile ducts were spreading from the periphery of the lobules around the portal septa. The lining epithelium of the septal bile ducts was columnar or cuboidal and consisted of lightly bluish stained protoplasm containing large, pale vesicular nuclei. In some of these, mitotic figures were observed.

Seven days after excision the number of necrotic areas in the stage of progressive absorption was diminished. Anisocytosis and anisokaryosis were still characteristic features of most liver cells. In contrast to the coarse granulation of normal liver cells, hepatic cells at this stage showed a very fine granular cytoplasm. The nuclei were large and frequently contained multiple nucleoli. Mitotic figures also were numerous. Characteristic for these livers was the markedly increased number of interlobular and intralobular bile ducts, apparently still in a stage of progressive formation. The portal septa contained many dilated bile ducts, while the newly formed ducts were aggregated in the areas surrounding the septa. In many of these areas microscopic changes were observed which indicated that hepatic cells might undergo transformation and proceed to the formation of intralobular bile ducts. The staining qualities of such cells gradually changed and the protoplasm became more homogeneous and hyaline. The size of the cells had diminished slightly and a number of them exhibited an acinar orientation around dilated bile canaliculi (Fig. 3). Concomitantly the sinus endothelium in these areas became prominent and generally it appeared

as if vascularity was increased in these areas. The demarcation of these acinar or ductal formations by the hyperplastic endothelium made them clearly distinguishable from the surrounding liver cell cords.

Fourteen days after excision new necrotic areas had been added. The liver parenchyma was about evenly divided between deeply stained liver cell cords and lightly stained bile ducts (Fig. 4). Anisokaryosis was pronounced. The nuclei were rich in chromatin, large, and showed many intranuclear fragments. Mitotic figures were seen in the newly formed bile ducts. Around many of these ducts a thin layer of fibrous tissue had developed.

Twenty-eight days after excision of the duct, hepatic necrosis was extreme. The remaining liver cells were arranged in acinar patterns around spaces which represented dilated bile canaliculi (Fig. 5). The fibrous tissue around the ducts was increased. Mitotic figures in liver cells and bile duct cells were substantially decreased.

Group II

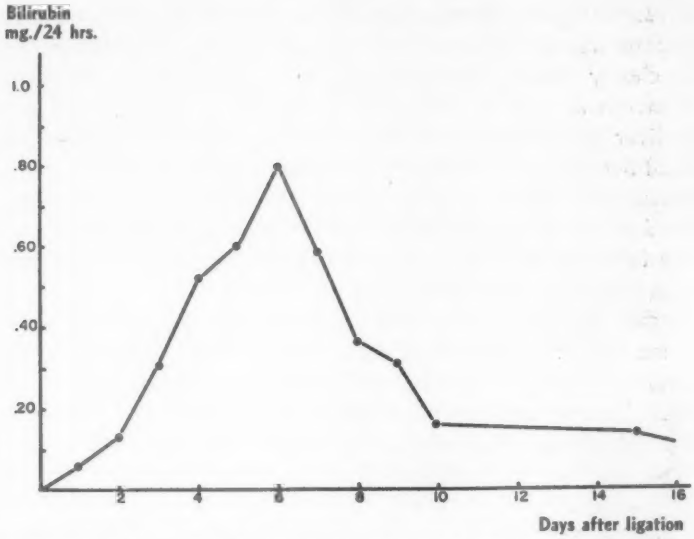
Identical symptoms were observed in rats with ligation of the common bile duct as in those of group I in the period from 7 to 14 days after the ligation. From then on, however, the behavior of the rats with ligated ducts was in striking contrast to that of the animals with excised ducts. Generally, 14 days after the ligation of the common bile duct the animals showed an increase in body weight, the discoloration of the skin disappeared, food intake increased, and urine output and color reverted to normal. Twenty-eight days following ligation all animals appeared healthy and showed no evidences of dysfunction.

These findings led to a more detailed investigation of the morphologic and functional changes induced by simple ligation of the common bile duct, as contrasted to excision of the duct.

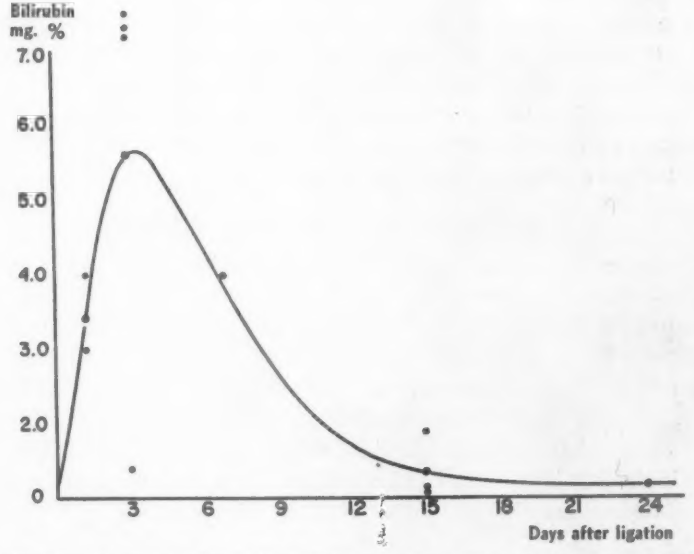
Laboratory Findings in Duct-Ligated Animals

Over a period of 3 to 10 days following ligation of the bile duct the animals showed a marked increase in urinary bilirubin (Text-fig. 1). Ten to 14 days after ligation the amount of urinary pigment decreased until only traces of pigment were present 28 days after ligation.

As shown in Text-figure 2, the values for blood serum bilirubin, as determined by the indirect diazotization, followed a pattern similar to that observed for urinary bilirubin, with a rapid increase of pigment within the first week after ligation followed by a more gradual decrease approaching normalcy at 28 days after the operation.



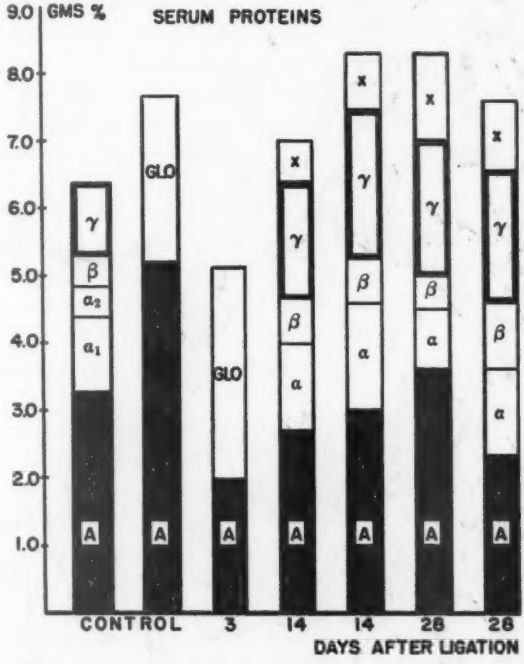
Text-figure 1. Urinary bilirubin in mg. per 24 hours after ligation of the common bile duct of the rat.



Text-figure 2. Serum bilirubin in sacrificed rats after ligation of the common bile duct.

Assay of urinary proteins in ligated rats showed that there was an increase in urinary albumin for 5 to 10 days after ligation. As much as 250 to 300 mg. of albumin were excreted in 24 hours by some animals.

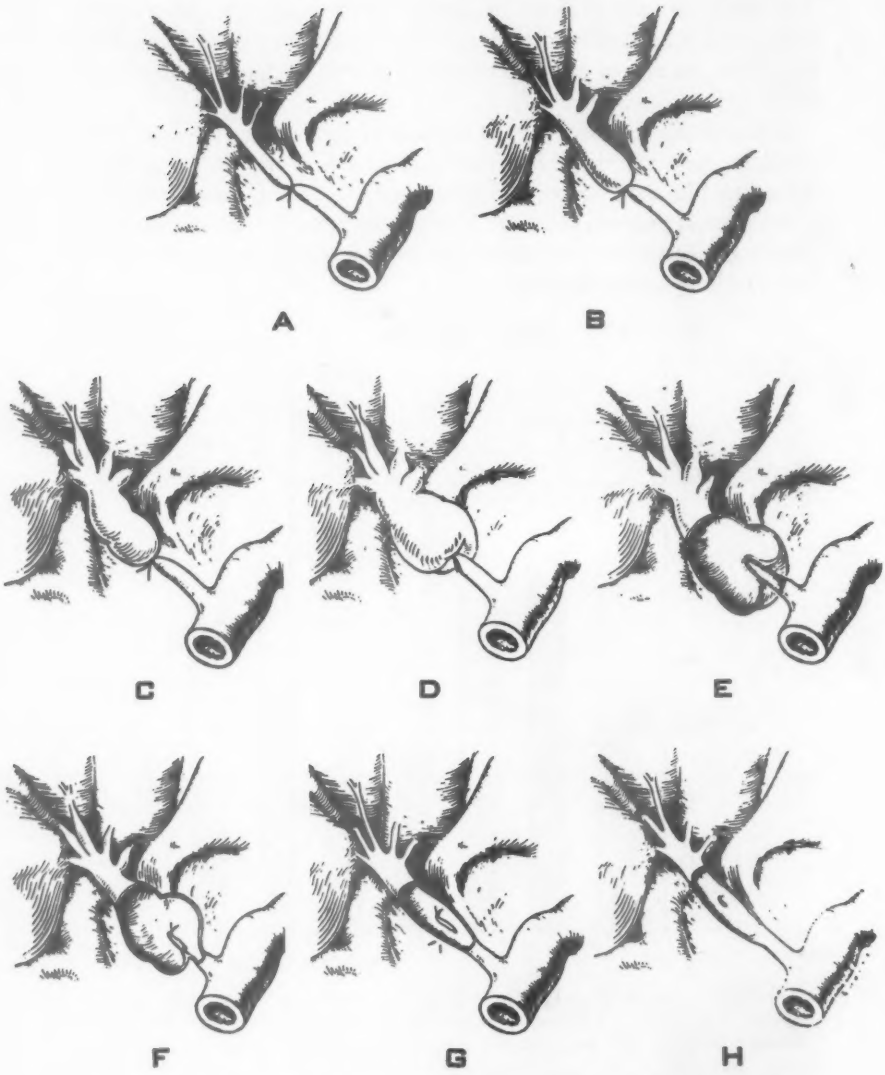
Representative values for the amount of the different serum protein fractions, as separated iontophoretically, are shown for a group of ligated rats in Text-figure 3. The most notable change was the increase in the gamma globulin fraction. It was noted that the albumin-globulin ratio was decreased soon after ligation and remained inverted for as long as 28 days after ligation.



Text-figure 3. Serum proteins in normal rats (controls) and in rats after ligation of the common bile duct. X indicates an unidentified slow-moving serum component. Albumen and globulin are indicated by A and Glo; Globulin fractions, by α_1, α_2, β, γ.

Gross Necropsy Findings

Up to 14 days after simple ligation of the common bile duct the findings were similar to those described for group I. In animals which survived this period—in this study, all but one—a gradual decrease of gross pathologic findings was noted. Fourteen to 28 days after ligation the proximal stump was only moderately dilated and at the later stages



Text-figure 4. Schematic illustration of eight stages in the mechanism of recanalization after ligation of the common bile duct in the rat. Dilation of the proximal portion of the duct. Invagination of the distal stump into the dilated proximal portion. Fusion of adjacent membranes with subsequent thinning out of the fused membranes and rupture into the distal stump. A patent common bile duct is re-established. Inside the still moderately dilated recanalized common duct the original ligatures frequently are found.

only minimal enlargement was observed. The liver appeared normal or only slightly enlarged *28 days* after ligation. All other organs were normal grossly. Careful dissection of the dilated bile duct and the surrounding areas showed that recanalization of the common duct had taken place. Trypan blue injected into the dilated duct appeared at the duodenal papilla in all instances in which return to normalcy was observed in duct-ligated rats. Studies carried out on the mechanism of recanalization revealed that invagination of the distal stump into the dilated proximal stump first took place. This was followed by a fusion of the adjacent membranes. Subsequently a part of the now fused stumps thinned out or became necrotic and the proximal stump ruptured into the distal end of the ligated duct. The original ligatures, placed around the common duct at the time of operation, frequently were found inside the still moderately dilated and now patent common bile duct. This process of recanalization following simple ligation of the common bile duct is illustrated schematically in Text-figure 4.

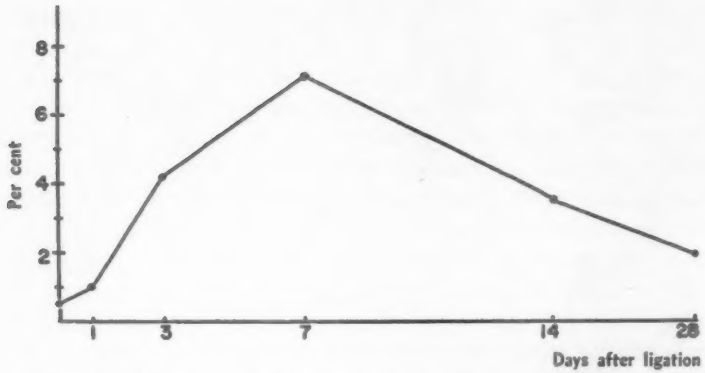
Microscopic Findings

Up to the 14th day after ligation of the common duct the microscopic changes resembled those found in the duct-excised animals of group I. Except for a few animals in which recanalization of the duct was delayed or had not taken place before sacrifice, all histopathologic changes in the recovering rats were reversed in the order in which they occurred.

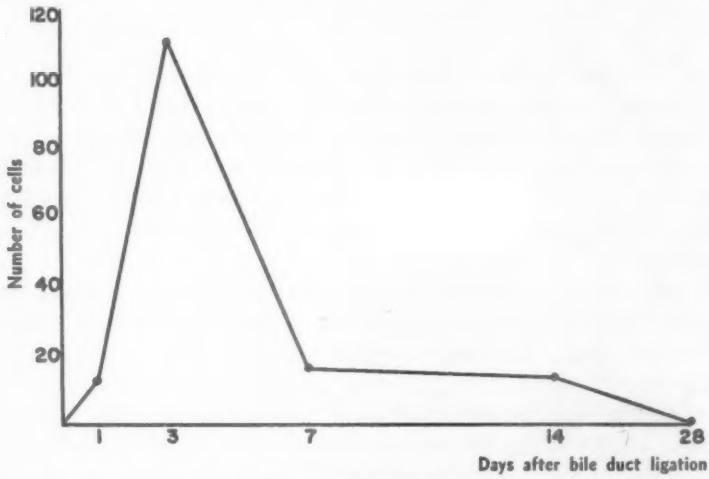
Fourteen to 28 days after ligation the newly formed bile ducts were regressing slowly. The cuboidal lining of the ducts became flattened and in many areas the lumen had collapsed.

Frequently the only remaining evidence of previous interlobular or intralobular bile duct proliferation consisted of fine strands of fibrous tissue. In rats which had recovered early after ligation, microscopic restitution of the liver was complete and no remnants of previous parenchymatous changes were evident. In animals in which recanalization was delayed, the restitution to normal appeared to be established slowly. In some, the portal spaces remained slightly enlarged.

A quantitative estimate of the observed microscopic changes by the Chalkley method[5] confirmed the impression that the changes in functional activity and in morphologic pattern in the liver were in good agreement in rats in which the bile duct had been ligated. Text-figures 5, 6, and 7 present average values obtained from the enumeration of different cellular units in the liver by this method.



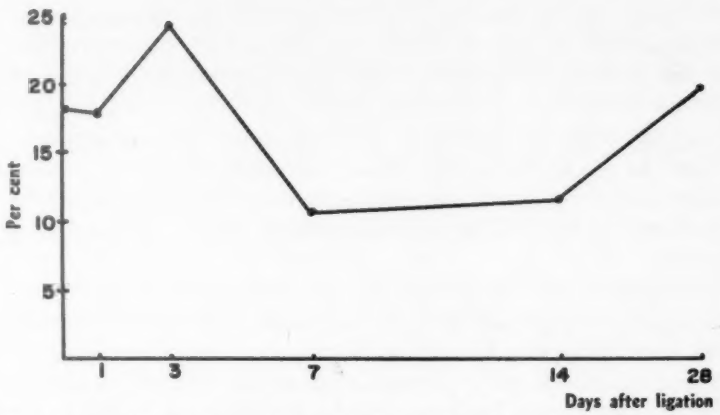
Text-figure 5. On the vertical axis are plotted the relative numbers of intralobular bile duct cells expressed as a percentage of the hepatic cord cells in the same areas.



Text-figure 6. The values on the vertical axis indicate the number of liver cells with mitotic figures per 100 high-power (× 430) fields.

Discussion and Conclusions

The strikingly different response to the two surgical procedures was of interest. While the excision of the common bile duct in animals of group I led to functional and morphologic changes which progressed to irreversible hepatic insufficiency and death, the animals of group II with ligation of the common bile duct initially showed the same pattern but eventually recovered. The laboratory data and the histopathologic changes as well as the evaluation of the hepatic parenchymatous



Text-figure 7. The relative number of liver cells with multiple nuclei is expressed as a percentage of mononuclear liver cells.

changes by the Chalkley method[5] reflect very well the pattern of an acute hepatic disorder and the subsequent return to normalcy often seen clinically with obstructive jaundice. While serum and urine bilirubin and the hepatic parenchyma were essentially normal at the end of the observation period of 28 days, the still elevated albumin-globulin ratio and the increased serum gamma globulin may indicate that recovery is not complete at that time.

Survival of rats after ligation of the common bile duct has been reported by other investigators[6]; no explanation was given, however, in these communications for the mechanism involved. The restitution of the ligated bile duct by invagination of the distal stump into the proximal stump with recanalization, as described, constitutes a remarkable biologic phenomenon. With a uniform ligation procedure more than 95 per cent of rats survive. This is in agreement with previous observations.[3] This operation may well serve as a tool to the laboratory investigator who wishes to study hepatic damage and recovery in the same animal.

In animals in which the common bile duct was partially excised, the progressive proliferation of newly formed bile ducts into the hepatic lobule, observed also by other investigators,[6-8] was impressive. In these animals the alterations in the hepatic parenchyma proceeded to an extreme degree. There was an almost complete change of hepatic parenchyma into a conglomerate of newly formed intralobular bile ducts, duct-like formations, and extensive areas of necrosis.

Microscopic study of the hepatic changes taking place after bile duct

excision as well as after bile duct ligation led to the impression that the newly formed intralobular bile ducts arose not only by mitotic division of duct epithelium but also by a metaplastic process from hepatic cells. Mitotic figures in liver cells were found in increasing numbers from the third day after the operation. The peak of mitotic activity was observed to be at the 7-day period. In accordance with a previous observation,[9] the increase of mitotic activity was accompanied by an increased number of liver cells in which the nuclei contained multiple nucleoli.

Although in rats in which there was spontaneous recanalization of the ligated common bile duct, the masses of newly formed intralobular bile ducts disappeared nearly as fast as they had appeared, reversal of duct epithelium into hepatic cells was not evident. It is possible that the disappearance of the many intralobular duct cells is due merely to atrophy and complete degeneration of the ductal formations.

Summary

The common bile ducts of adult male rats were excised or ligated. Excision led to hepatic insufficiency and death within 4 weeks after the operation. At that time, extensive hepatic necrosis and transformation of the hepatic parenchyma into bile duct-like patterns had occurred.

Ligation of the common bile duct resulted in recanalization and restitution of the bile duct. Functional and morphologic liver changes and the return to normalcy were studied in these rats. The mechanism, by which restitution of the ligated common bile duct was accomplished, was demonstrable in the material examined.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

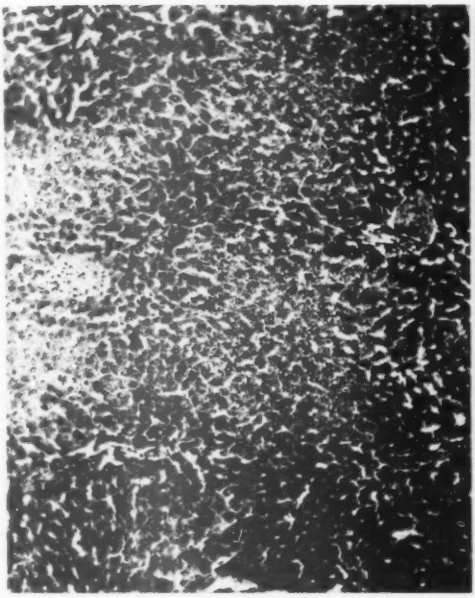
FIG. 1. Liver of a rat 14 days after excision of part of the common bile duct. The proximal stump is markedly dilated. The liver is enlarged and discolored.

FIG. 2. Liver of a rat 1 day after excision of the common bile duct. The central portions of the lobules are weakly stained, in contrast to the periphery which stains heavily. Two small necrotic areas can be seen in the parenchyma. Hematoxylin and eosin stain. × 40.

FIG. 3. Liver of a rat 7 days after excision of the common bile duct. Acinar orientation of liver cells around dilated bile canaliculi. The protoplasm is homogeneous and less granular. The surrounding sinus endothelial cells are prominent, showing some concentric orientation. The liver cell nuclei are large and vesicular. This area is representative of early transformations into intralobular bile ducts. Hematoxylin and eosin stain. × 400.

FIG. 4. Liver of a rat 14 days after excision of the common bile duct. A large portion of the parenchyma is replaced by newly formed bile ducts. Hematoxylin and eosin stain. × 40.

FIG. 5. Liver 28 days after excision of the common bile duct. Necrosis is progressive. The remaining liver tissue consists mainly of newly formed bile ducts and of liver cells arranged in an acinar pattern around markedly dilated bile canaliculi. Hematoxylin and eosin stain. × 40.



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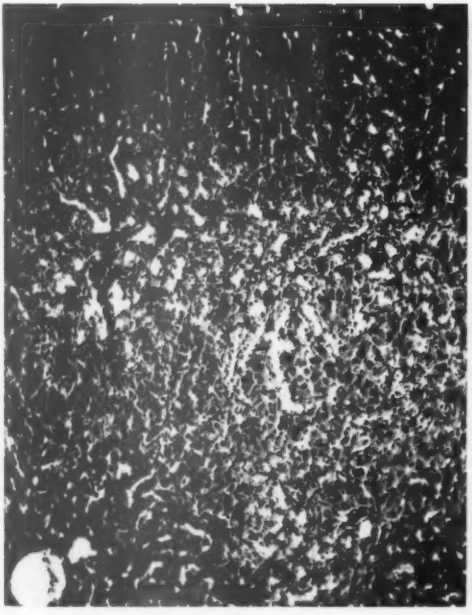

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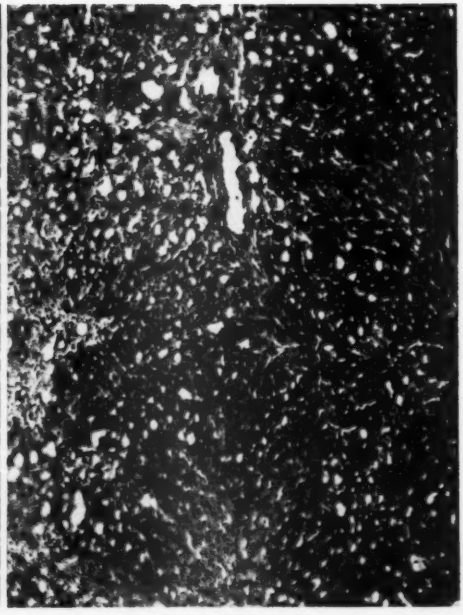

5

HEPATIC FIBROSIS AND CIRRHOSIS IN MAN IN RELATION TO MALNUTRITION *

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Since a deficiency of certain nutrients may cause hepatic damage in experimental animals,[1-3] the possibility has been considered that the high frequency of hepatic lesions in malnourished communities may be the direct result of similar deficiencies.[1-8] In view of the economic implications of this hypothesis, and since recent studies have cast doubt on such a simple relationship,[9-12] we are presenting our observations on the development of liver disease in a group of South African Bantu, whose diet is regarded as inadequate by orthodox standards. In general, our findings add support to the view that the rôle of diet in liver disease among adults is far from established in such communities.

MATERIAL AND METHODS

Our material was drawn from 876 consecutive necropsies on Bantu patients dying at Baragwanath Hospital (1,500 beds), the majority of whom were domiciled in Johannesburg or surrounding townships. An average of two sections from the liver were available for review in all but 20 cases (2.3 per cent). In these, however, the necropsy records stated that the organ was normal.

In addition, 215 specimens of liver were taken for biopsy. In general, these were derived from patients in whom liver disease was suspected.

All sections were stained routinely with hematoxylin and eosin. Additional stains used when desired were Masson's trichrome stain for collagen and a silver reticulin stain. Alcoholic hyalin was demonstrated by Mallory's stain, iron pigment by Perls's reaction, and fat by oil red O or Sudan IV on frozen sections.

To simplify the classification of liver disease, the following groupings were adopted:

Group I. Non-Fibrotic Livers. Livers which showed no increase in portal fibrosis, or only slightly increased collagen or reticulin in an occasional portal triad.

Group II. Slight Portal Fibrosis. Livers in which there was a slight

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but definite diffuse increase in fibrosis affecting the majority of the portal triads.

Group III. Moderate Portal Fibrosis. Livers similar to those in group II, but in which the portal tracts tended to be linked by fibrous tissue ("peri-portal fibrosis"). In groups II and III the normal lobular architecture was, in general, preserved as represented by the relationship of portal tract to central vein (Fig. 1).

Group IV. Severe Diffuse Septal Fibrosis. An advanced degree of group III fibrosis with intralobular fibrosis causing lobular distortion, but without significant parenchymal cell hyperplasia or formation of regenerative nodules ("mild cirrhosis") (Fig. 2).

Group V. Severe Cirrhosis. Livers with marked distortion of architecture due to scarring and the formation of regenerative and hyperplastic parenchymal nodules (Figs. 3, 9, and 10).

Group VI. All livers from patients in whom death was primarily due to hepatic necrosis. Scattered areas of focal necrosis such as are seen in enteric fever and generalized sepsis were not included, and such livers were classified according to the degree of fibrosis.

Group VII. Group VII included livers in which no satisfactory grouping could be attempted because of irregular fibrosis or massive tumor infiltration.

Focal fibrosis due to tuberculosis, bilharzial granulomas, or abscess formation was ignored, as was also centrilobular fibrosis.

Grading of Fatty Change. Livers were graded from 0 to 3 plus according to the proportion of cells containing fat droplets (i.e., 0, < 30 per cent, 30 to 60 per cent, and > 60 per cent).

Grading of Hepatic Siderosis. The grading of hemosiderin deposition (0 to 3 plus) was the same as that used previously.[13]

Development of Portal Fibrosis and Cirrhosis in the Bantu Liver

The age distribution of each type of hepatic lesion is shown in Tables I and II. For convenience, lesions will be discussed under the following headings:

Diffuse Portal Fibrosis (Groups II to IV)

Diffuse portal fibrosis was an incidental post-mortem finding and no evidence of portal hypertension was observed. In only 2 of the 15 livers with severe diffuse fibrosis (group IV) did the hepatic lesion appear related to the cause of death. While the frequency and severity of fibrosis increased with age in both sexes, it commenced earlier and was more severe in males than in females.

Many livers with moderate or severe fibrosis showed a fine surface granularity, with accentuation of the lobular markings on section. No constant relationship was apparent between weight of the liver and degree of fibrosis.

TABLE I

Percentage Distribution of Hepatic Lesions in Bantu Males According to Type and Age Groups *

Hepatic lesion	0–4 yrs.	5–14 yrs.	15–24 yrs.	25–34 yrs.	35–44 yrs.	45–54 yrs.	55–64 yrs.	65+ yrs.	All ages
	%	%	%	%	%	%	%	%	%
I No fibrosis	90.7	72.3	48.2	51.9	51.2	36.6	34.0	23.2	51.7
II Slight portal fibrosis	8.2	11.2	29.6	27.9	25.2	38.7	32.0	41.8	27.1
III Moderate portal fibrosis			3.7	5.1	9.4	11.8	20.7	25.6	9.5
IV Severe septal fibrosis				1.3	2.4	4.3	3.8		1.9
V Cirrhosis		11.2	11.1	10.1	10.2	6.5	7.6	7.0	7.4
VI Necrosis		5.5	3.7	1.3	0.8				0.8
VII Miscellaneous, unclassified	1.2		3.7	2.6	0.8	2.1	1.9	2.3	1.5
Number in each group	85	18	27	79	127	93	53	43	525

* The degree of siderosis and of fatty change was ignored in this classification.

TABLE II

Percentage Distribution of Hepatic Lesions in Bantu Females According to Type and Age Groups *

Hepatic lesion	0–4 yrs.	5–14 yrs.	15–24 yrs.	25–34 yrs.	35–44 yrs.	45–54 yrs.	55–64 yrs.	65+ yrs.	All ages
	%	%	%	%	%	%	%	%	%
I No fibrosis	89.9	83.4	83.8	83.9	73.8	54.5	57.1	57.6	74.1
II Slight portal fibrosis	10.1	8.3	13.5	10.7	13.1	27.1	32.1	12.1	15.7
III Moderate portal fibrosis		8.3†		1.8	6.6	7.3	3.6	18.2	4.8
IV Severe septal fibrosis					1.6	3.6	3.6	3.0	1.4
V Cirrhosis					3.3	7.3	3.6	6.1	2.6
VI Necrosis			2.7	1.8					0.6
VII Miscellaneous, unclassified				1.8	1.6			3.0	0.9
Total number in group	69	12	37	56	61	55	28	33	351

* The degree of siderosis and of fatty change was ignored in this classification.

† A case of rheumatic heart disease with well marked chronic venous congestion. Both central and portal fibrosis were present.

In mild fibrosis, on microscopic examination, the portal triads showed an increase in reticulin and/or collagen fibers and membranes. Such triads were often infiltrated by lymphocytes, histiocytes, and

plasma cells; also by occasional neutrophils and eosinophils. In some livers, however, the picture was that of an inactive portal fibrous scar, cellular infiltration and fibroblastic activity being minimal or absent. In more marked portal fibrosis, the lamina limitans was often disrupted by radiating collagen septa surrounding isolated and degenerating liver cells. In a number of livers, newly formed bile ducts were seen in the portal triads, the appearances of which suggested cholangiolar proliferation. Although diffuse, fibrosis tended to be irregular and to involve the triads to a varying degree (Figs. 1 and 2).

A prominent feature in many livers was the presence of heavy hemosiderin deposits in the liver cells, Kupffer cells, and portal triads (Figs. 1 and 2). In these livers, fibrosis, new bile duct formation, and inflammation tended to be prominent. In addition, many showed foci of parenchymal cell degeneration, often in the region of the central vein associated with infiltration by hemosiderin-laden macrophages. Fibrous and reticulin membranes were observed in such foci, and by linking with the portal triads appeared to be the pathway by which the diffuse septal fibrosis seen in group IV livers developed (Figs. 4 and 5). However, the basic portal nature of the fibrosis usually could still be detected. Although architectural distortion was present in such livers (group IV), evidence of parenchymal regeneration or hyperplasia was slight, and the formation of pseudolobules was rare. Further, the absence of portal hypertension in such cases did not suggest widespread regeneration.

Severe Cirrhosis (Group V)

Hepatic carcinoma was observed in 21 (44 per cent) of the 48 livers in this group. Of the remaining 27, evidence of portal hypertension was present in 19. Cirrhosis of this type was found more frequently in males than in females, but the incidence was unrelated to age; or, if anything, was more common in the younger ages, in contrast to diffuse fibrosis.

While all livers in group V, by definition, showed cirrhosis with nodular hyperplasia, in some the lesions were more diffuse and less irregular than in others. Accordingly, an attempt was made to classify these livers morphologically. In 5 livers moderate fatty change (2 plus and 3 plus) with micro-cyst formation was present and the cirrhosis appeared to be of a diffuse septal type. In 2 of these, alcoholic hyalin was prominent. These 5 livers were, in general, similar to those described as "alcoholic cirrhosis" in white races. In one liver the his-

tologic picture suggested a group IV liver with diffuse parenchymal hyperplasia. For 3 livers no definite conclusion was reached.

In the remaining 39 livers, the hyperplastic nodules were more irregular in size and the surface showed greater nodularity. Of 18 non-neoplastic livers, 9 weighed 1,200 gm. or less, and only 3 more than 2,000 gm.

Microscopically, these livers showed hyperplastic parenchymal nodules, sometimes necrotic and bile stained, surrounded by bands of condensed stroma and fibrous membranes, often containing degenerating liver cells and numerous newly formed small bile ducts. In many livers these bands were of considerable width and suggested massive parenchymal collapse (Figs. 3 and 6). Infiltration of the stroma by lymphocytes and histiocytes was usually marked, but neutrophils, eosinophils, and plasma cells sometimes were observed. Fatty change and alcoholic hyalin were not evident. The most significant feature was the irregularity of the changes, since some areas were relatively unaffected by collapse or hyperplasia, the liver architecture being essentially normal. Using the criteria proposed by Mallory,[14] Himsworth,[1] and Goldblatt,[15] the general pathologic picture suggested post-necrotic cirrhosis.

Other Lesions

Hepatic Necrosis. Six livers showed acute or subacute necrosis. In 4, the necrosis was massive and the features were similar to those described in fulminating viral hepatitis[16] (Fig. 7). The remaining 2 showed diffuse central necrosis.

Fatty Change. Infants and Children. In this series fatty change was seen constantly only in infants suffering from kwashiorkor or severe gastro-enteritis. Of the 154 livers from patients under 5 years, 27 (17.5 per cent) were from cases of kwashiorkor. The histologic picture was similar to that described by others,[17] the organ showing marked fatty change with micro-cyst formation and cellular infiltration of portal triads (Fig. 8). Mild portal fibrosis, however, was seen in only 3 cases. In male infants between 1 and 2 years, only 1 of 13 kwashiorkor livers, but 3 of 12 non-kwashiorkor livers showed minimal fibrosis. In 2 livers from patients who had recovered from one or more previous attacks of kwashiorkor, no significant fibrosis or reticulin formation was noted. Not one kwashiorkor liver showed massive necrosis or post-necrotic scarring.

Adults. Fatty change was rare in adults and was not a feature of livers showing diffuse fibrosis. Slight fatty metamorphosis, usually

in females, was, however, found in chronic disease of extrahepatic origin, as in white people. Only 3 large fatty livers with fibrosis of the type described as "subacute portal cirrhosis" by Hall *et al.*[18] were observed, 2 being associated with generalized tuberculosis. Of the 215 liver specimens examined from adults, severe fatty metamorphosis (2 plus or 3 plus) was found in only 12, of which 9 were from women.

Siderosis. The histopathology of siderosis in the Bantu of Southern Africa has been discussed elsewhere.[13] In this series, the association of moderate and severe siderosis (2 plus and 3 plus) with portal fibrosis is shown in Table III; the proportion of siderotic livers in-

TABLE III

Relationship of Diffuse Portal Fibrosis to Other Pathologic Lesions

Associated disease or lesion	Degree of fibrosis					
	Males			Females		
	Mild II	Moderate III	Severe IV	Mild II	Moderate III	Severe IV
Moderate or severe siderosis	77	41	10	10	13	5
Miliary tuberculosis	18	1	1	3	4	1
Chronic venous congestion	48	15	2	22	6	
Chronic wasting disease	9	6		5	2	
Chronic gastro-intestinal disease	5	2		1		1
Chronic hepatitis or cholangiolitis	2	1	1	4		1
Amebic abscess or enteric fever	5	1		4		
Schistosome ova	4					
Kwashiorkor	2			1		
No obvious lesion or disease	16			9	2	
Total number of livers in group*	142	50	10	55	17	5

* This represents the total number of livers in the group, not the total number of lesions. When livers showed both siderosis and another lesion they have been entered for both.

creased with the severity of the fibrosis, siderosis being present in all livers with group IV lesions. But, as formerly reported,[13] siderosis was observed also in a small proportion of non-fibrotic livers. It is of interest that if livers with significant siderosis (2 plus or 3 plus) are excluded, the incidence of diffuse portal fibrosis in both sexes is similar (14.2 per cent in males and 13.9 per cent in females).

There was no demonstrable association between siderosis and severe cirrhosis.

Primary Carcinoma of the Liver. There were 26 primary carcinomas of the liver in this series, 24 in males and 2 in females. All were of the liver-cell type, with the exception of one of bile-duct-cell type in

a female. In males, 21 (87 per cent) tumors arose in livers with severe cirrhosis (group V). While 54 per cent of these livers developed malignant change, none of the 9 such livers in females did so, which is significant ($P < 0.02$). The remaining tumors arose in livers with diffuse portal fibrosis. The histologic appearance in many cirrhotic livers with malignant change suggested that the carcinoma developed from hyperplastic parenchymal nodules, many of which showed marked atypicality and hyperplasia and at times appeared to merge with the tumor (Figs. 9 and 10).

Hepatic Disease and Biochemical Dysfunction

Local studies indicate that children who have recovered from kwashiorkor may be left with residual hepatic dysfunction as reflected by biochemical tests, despite normal histologic findings on liver biopsy.[19-21] Furthermore, children who have had no overt attack of kwashiorkor may also develop similar abnormalities.[20,22]

To what extent the biochemical changes persist into adult life is uncertain, but over two thirds of young adult Bantu reveal abnormalities[23,24] although we have found no significant hepatic lesion at biopsy in a number of such cases. It is clear, therefore, that a high proportion of livers in adults cannot be regarded as metabolically normal.

Discussion

Summary of Pathogenesis of Hepatic Disease in the Bantu

In infancy and early childhood, the most common hepatic lesion was the severe fatty change seen in kwashiorkor.

In adolescence and early adult life, most livers appeared relatively normal, apart from a small number showing mild portal fibrosis, usually associated with tuberculosis or chronic venous congestion. Isolated cases of acute hepatic necrosis and post-necrotic scarring were seen also.

In adults, hepatic lesions were of two major types: (1) A diffuse portal fibrosis which, in the more severe cases, was associated with well marked siderosis. The age distribution and histologic pattern in many livers suggested a low-grade progressive chronic cholangiohepatitis. (2) A severe cirrhosis which, in the majority of instances, suggested a post-necrotic origin and which occurred with approximately equal frequency throughout life. The different age distribution and pathologic patterns of these two lesions suggested that they were not related. Further, the age gap between the fatty liver of kwashiorkor and appearance of the more severe degrees of diffuse portal fibrosis indicated

that the latter was not a direct sequel of kwashiorkor. Too great reliance should not, however, be placed on the etiologic distinction made between the various types of severe cirrhosis (group V), in view of Dible's[25] caution on the frequent impossibility of determining the morphogenic pathway by the final picture.

Nutrition: General Remarks on Human Malnutrition

Animal Studies and Their Limitations. Liver disease in rats and other animals caused by dietary manipulation has been amply studied. Such experimental diets include those deficient in protein, in various amino-acids and vitamins, and also in certain lipotropic factors.[1,2] Excess of other nutrients such as cystine, riboflavin, and biotin, also may be harmful in certain dietary contexts.[2] It must be remembered, however, that a diet satisfactory for humans is apparently inadequate when fed to rats and monkeys.[26,27] In man deficiencies of nutrients almost invariably are multiple, and many other factors such as bacterial infection, parasitic infestation, and climatic stress may be of importance. Very little is known for man of the possible adaptations in metabolic activity and the physiologic requirements of nutrients which may develop in a specific dietary context, a fact of possible significance when discussing diets differing markedly from those of Western populations. The human requirements or even the need for such dietary factors as cystine, choline, inositol, biotin, and alpha-tocopherol are not yet established.

These considerations increase the difficulty of determining whether the Bantu diet is adequate in respect to particular nutrients. Nevertheless, in view of the misconceptions prevailing, it seems worthwhile to assess the adequacy of their diet in respect to certain nutrients in terms of orthodox beliefs in nutritional requirements.

Nutritional Consideration with Bantu Infants and Young Children. There is evidence that the composition of Bantu breast milk,[28,29] and the growth and general health of exclusively breast-fed Bantu babies compare favorably with their white counterparts.[30] At weaning, however, which is often sudden, babies are given a variety of foods, mainly cereal paps, but also proprietary cereal and dried-milk preparations, which are usually diluted excessively.[31] Such a diet, in comparison to breast milk, while adequate in methionine, is usually deficient in every other essential amino-acid as well as in total protein. In addition, when judged by amounts recommended for infants, the diet will be deficient in fat, calcium, vitamin A, riboflavin, niacin, possibly thiamine, and vitamins C and D. The amounts of choline, inositol, and

vitamin E will depend on the type of milling of the cereal; if the cereal is refined, the concentration of these nutrients will be very low. It is with this grossly inadequate dietary pattern that kwashiorkor so frequently occurs, and understandably the inadequacies are not remedied by supplements of single amino-acids, lipotropic substances, or vitamins.[17]

Nutritional Considerations with Bantu Adults. Information available from recent dietary surveys in Johannesburg and other areas[32-35] indicates that the adult Bantu diet, speaking generally, while markedly different in pattern from those of Europe and America, is seriously deficient in neither total protein nor amino-acids, in so far as the minimum requirements suggested by Rose[36] and associate workers are applicable, a conclusion reached also by Patwardhan[12] for tropical and subtropical peoples elsewhere. Unfortunately, dietary surveys usually provide average figures only for population groups and many individuals must consume much less than the mean figures reported. However, they do permit assessment of the probability of deficiencies of certain nutrients, the lack of which are known to lead to liver disease in various animals.

Etiology of Liver Lesions

Kwashiorkor

The possible etiologic factors causing kwashiorkor have been reviewed recently.[37] There is now general agreement that the syndrome is a manifestation of "protein malnutrition" in infants and young children. Recently, it has been demonstrated that cure may be initiated by a synthetic diet composed exclusively of eleven amino-acids.[38,39] While there is still uncertainty whether the liver changes are secondary to pancreatic damage, it appears justifiable to regard the fatty liver of kwashiorkor as a true example of human liver disease due to an inadequate diet. Yet, this series provides no evidence that progressive fibrosis, cirrhosis, or other sequelae develop directly in such livers, a conclusion similar to that reached in other local studies.[3,21]

Diffuse Portal Fibrosis

Diffuse portal cirrhosis in adults often has been called "nutritional or tropical cirrhosis" on the African continent. While fibrosis can scarcely be ascribed to the same cause in all livers, nevertheless, the histologic picture associated with the age trend (Tables I and II) suggests that a common morphogenic pathway is present in a large proportion. Notwithstanding, mild increased scarring and fibrosis of the portal triads may be observed occasionally in European subjects

dying without a history or other evidence of hepatic injury. It is probable that such lesions may result from non-specific hepatitis due to chronic intestinal disease, chronic bacterial infection, or heart failure.[40,41] The association of such diseases to portal fibrosis is shown in Table III and such possible factors cannot be ignored.

Protein and Lipotropic Agents. Fatty metamorphosis induced by deficiency of lipotropic agents in the rat may cause diffuse hepatic fibrosis which is non-portal in distribution. The fibrosis in the Bantu livers was predominantly portal and did not appear related to the fatty liver of kwashiorkor. Reviewing the dietary data available, we believe that there is no clear-cut evidence of gross deficiencies of total protein or of amino-acids (including methionine) in the local adult Bantu diet, although the major part of the ingested protein is from vegetable sources. In these surveys the range of means for the groups studied was 63 to 112 gm. of gross protein per diem per man unit. While the significance of choline has not been satisfactorily demonstrated in human nutrition, we have calculated that the average adult Bantu ingests an amount well within the range reported for white groups. Finally, the uncommonness of significant fatty infiltration in the adult livers also militates against the view that this latter lesion and, accordingly, lipotropic deficiency, were direct etiologic factors.

Alcohol. Liberal consumption of fermented cereal foodstuffs is widespread in South Africa.[34,42] Although the alcohol concentration is inconsiderable, the large volumes consumed probably entail an alcohol consumption reaching or even exceeding that of the drinking population of the United States. Fatty change, a characteristic feature of "alcoholic cirrhosis" in white adults,[40,41] was rare in this series. Further, in a series of 15 liver biopsies on alcohol-conditioned pellagrins, no significant fatty change was found. We hesitate, therefore, to regard alcohol as a factor of significance in the etiology of liver disease as seen here. It is possible, however, that the response to alcohol, directly or indirectly, in the Bantu pattern of diet is not identical to that seen with the European dietary context. The absence of fatty change may be analogous to the relative rarity of atherosclerosis in the Bantu.

Vitamins. In experimental studies on animals, thiamine and other members of the vitamin B complex have been implicated in the development of fatty change in the liver.[1,2] In the Bantu diet, calculated intakes of thiamine and total niacin usually are adequate, although intake of riboflavin may be marginal. The mean intake for vitamin A and its precursors in the Johannesburg survey in 50 families was 6,000 i.u. per man unit.[34]

Siderosis. It has been postulated that the widespread siderosis among the Bantu in Southern Africa is the result of excessive iron intake, which may be up to 200 mg. per diem.[43] Originally we regarded this iron deposition as harmless,[13] bearing in mind failure to produce fibrosis by experimentally induced siderosis. But in the present series, excluding livers with severe cirrhosis, the association of siderosis with the more severe degrees of diffuse portal fibrosis was marked (Table III). In many livers, foci of liver cell degeneration associated with infiltration by hemosiderin-laden macrophages and early fibrosis were noted (Fig. 5). Diffuse fibrosis and siderosis were more marked in males than females, but the incidence of hepatic fibrosis without significant siderosis was the same for both sexes.

Walters and Waterlow[9] have suggested that diffuse portal fibrosis in infants and children in West Africa is the result of the combination of malnutrition and malaria, the former predisposing the liver substrate to the noxious influence of the latter. It is possible that hemosiderin in the Bantu may act in a similar manner, its toxic effect being dependent on previous damage to the liver.

To summarize, in Table III is tabulated the relationship of diffuse portal fibrosis to siderosis and diseases believed to cause mild portal fibrosis. If these are regarded as possible etiologic factors, there are few cases of diffuse fibrosis in this series for which no possible cause can be postulated.

Severe Cirrhosis

Since the age distribution and histologic pattern in the majority of the cases with severe cirrhosis suggested post-necrotic origin, discussion will be limited to this group.

Nutritional Background: Protein and Amino-Acid Deficiency. In experimental animals, acute hepatic necrosis has been produced by protein and cystine deficiency accentuated by lack of alpha-tocopherol and factor 3.[2] Evidence from local dietary surveys[32-35] suggests, however, that the Bantu do not suffer from gross protein deficiency; moreover, maize, the staple foodstuff, is not deficient in cystine. The latter is not an essential amino-acid for man, nor have the human requirements or even need of alpha-tocopherol been demonstrated. It would seem unwise, therefore, to attribute the hepatic necrosis observed in the Bantu as due solely to nutritional deficiences of the type described in animals, nor is there evidence of an excessive intake of those factors known to enhance experimental hepatic necrosis.

Condiments, Toxic Drugs, and Chemicals. It is believed that the widespread and excessive use of condiments in India and Ceylon may

be a factor in promoting hepatic necrosis.[16] However, condiments are not a feature of the South African Bantu diet. These people do, however, consume a wide variety of native medicines, the composition and effects of many of which are unknown. From Baragwanath Hospital, Grusin[44] has described 5 cases of liver poisoning due to potassium bichromate, the latter sometimes being used as a daily emetic, and it is possible that other hepatotoxic drugs may be implicated. In this series only one case of Chiari's syndrome was observed, but no livers with the features of acute senecio poisoning, or veno-occlusive disease[45] were found, although we have since observed 4 such cases in infants. Therefore, the etiologic rôle of hepatotoxic drugs requires further investigation.

Hepatotoxic Viruses. In the absence of a satisfactory test for hepatotoxic viruses, their significance is difficult to assess, the investigator being mainly dependent on the histologic appearance of the liver and on the clinical history. "Infective hepatitis" is endemic in Johannesburg and about 30 Bantu patients with this diagnosis are admitted each year to Baragwanath Hospital. The mortality is approximately 3 per cent. In a 3-year period, 48 patients diagnosed as having "acute yellow atrophy" were admitted, with a mortality of 54 per cent. Some of these patients almost certainly had fulminating viral hepatitis, and the histologic pattern of the 4 livers with massive necrosis in the present series was consistent with such a diagnosis.

Although in Europeans, examples of post-necrotic scarring and primary carcinoma are described as following viral hepatitis, reports indicate that such a sequence is more common in Africans,[46,47] Singalese,[16] and Indonesians,[48] some workers attributing the high frequency of such sequelae to previous liver damage.[47] In this series, the number of livers with hepatic necrosis is small, but the age distribution is consistent with a relationship to post-necrotic cirrhosis. Further, the age distribution of the latter suggests a factor acting constantly throughout life, a rôle which a virus could fill. The possibility of a virus as a major cause not only of post-necrotic cirrhosis but also of liver cancer in this country thus needs investigation.

Undernutrition. Gross deficiencies of calories and nutrients lead to atrophy of the liver, as well as of other organs,[41] but fibrosis and cirrhosis are not a feature. In any case, the Bantu diet is seldom seriously deficient in calories, and the livers in the present series were not atrophic.

Parasites. Malaria is not endemic in the Johannesburg region. Mild bilharzial (*Schistosoma haematobium*) infestation, however, is wide-

spread, particularly among males in this area, but no significant correlation has been demonstrated between this parasite and diffuse hepatic fibrosis, cirrhosis, or liver cancer.[49]

Conclusions

It would appear that in childhood, abnormalities of liver metabolism may arise in the Bantu which are not necessarily reflected by histologic liver damage.[19-22] There is some evidence that these abnormalities persist into adult life,[23,24] and are irreversible. Thus Wayburne and Bersohn[50] found no significant alteration in the tests for liver function of trainee Bantu nurses followed for 2 to 3 years, despite consumption of an adequate diet; and we have previously reported a similar frequency of hepatic dysfunction in both poorly fed and in adequately fed Bantu subjects.[23] Furthermore, from Tables 19A and 19D in the study of Gillman and Gillman,[3] it would appear that the pattern of liver disease seen in severe malnutrition (pellagra) is similar to that in healthy Bantu adults dying accidently.

There is evidence in man and animals that a healthy liver is less susceptible to hepatotoxic agents than a dysfunctioning organ.[40] Accordingly, it would appear reasonable to suggest that the significance of malnutrition in this locality is dependent mainly on the damage caused to the liver in infancy and early childhood, which renders the organ, in comparison to that of the European, more susceptible to stresses in adult life.

The nature of these stresses is not established, but the present observations suggest that siderosis, hepatotoxic viruses, and possibly toxic drugs may be of major importance. If this hypothesis is acceptable, there is little necessity to regard the diet of the adult Bantu as a major factor in the causation of liver disease. This does not imply that amino-acid imbalance, with concomitant changes in metabolic pathways, is not of significance, nor do we exclude the probability that isolated individuals may suffer from gross dietary deficiency. But we do maintain that the high incidence of liver disease in the Bantu is far from being explicable on the basis of clear-cut and widespread nutritional deficiencies in these people.

Summary

The results of pathologic examination of the livers from 876 consecutive necropsies and of specimens of liver from 215 biopsies on South African Bantu patients are reported.

Hepatic lesions were found to be of three major types: (a) A fatty liver occurring in infancy due mainly to kwashiorkor and rarely fol-

lowed by fibrosis and necrosis; (b) a fine symptomless portal fibrosis, most frequent in the second half of life and usually associated with heavy hemosiderin deposition; and (c) a severe cirrhosis, most frequently of post-necrotic origin and showing no specific age trend. More than half of the livers of the third group had developed primary malignant proliferation.

The causative rôle of dietary and non-dietary factors in the development of these lesions is discussed. Apart from severe malnutrition (kwashiorkor) in infancy, there is no adequate evidence that the nutritional factors which so readily induce hepatic damage in small animals are involved; in particular, it is doubted whether gross deficiencies of protein and amino-acids may be invoked as etiologic factors for the hepatic lesions so common among the Bantu.

In infancy, however, widespread liver damage, although not usually recognizable histologically, is demonstrable by various biochemical tests of liver function, and it is probably caused by malnutrition. It is suggested that such damage predisposes the liver to factors which, in adult life, precipitate liver damage. For the Bantu, it seems probable that such factors include siderosis, hepatotoxic viruses, and possibly toxic drugs.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Liver (group III) from a 52-year-old man who died from lobar pneumonia. Moderate portal fibrosis with early linking up of the portal triads, but with preservation of the normal relationship of the portal triads and central veins. Heavy hemosiderin deposits also are present. Hematoxylin and eosin stain. × 40.

FIG. 2. Liver (group IV) from a 50-year-old woman who died of scurvy. Severe portal fibrosis with loss of the normal relationship of the portal tract to the central vein due to fibrosis. Marked hemosiderin deposits also are present. Hematoxylin and eosin stain. × 40.

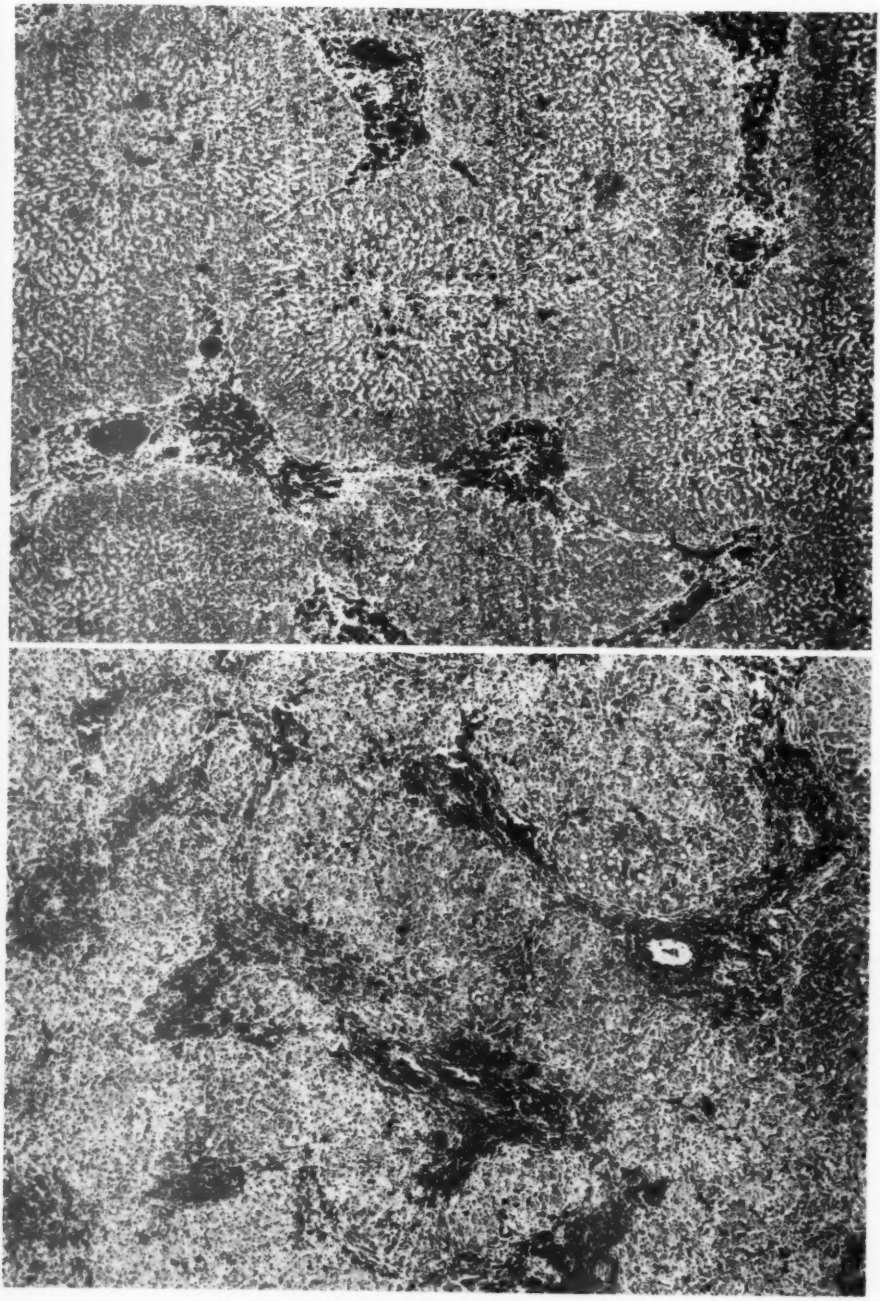

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FIG. 3. Severe cirrhosis (group V) with large areas of condensed stroma and fibrosis in which degenerated and necrotic liver cells are present. Small islands of regenerating parenchymal cells also are present. Hematoxylin and eosin stain. × 30.

FIG. 4. Liver obtained for biopsy, showing large clumps of hemosiderin in the parenchyma associated with degeneration of liver cells. Reticulin stain. × 150.

FIG. 5. A focus of hemosiderin deposited in sinusoids, with increase in reticulin membranes due to degeneration of liver cells. Same liver as that seen in Figure 4. Reticulin stain. × 600.

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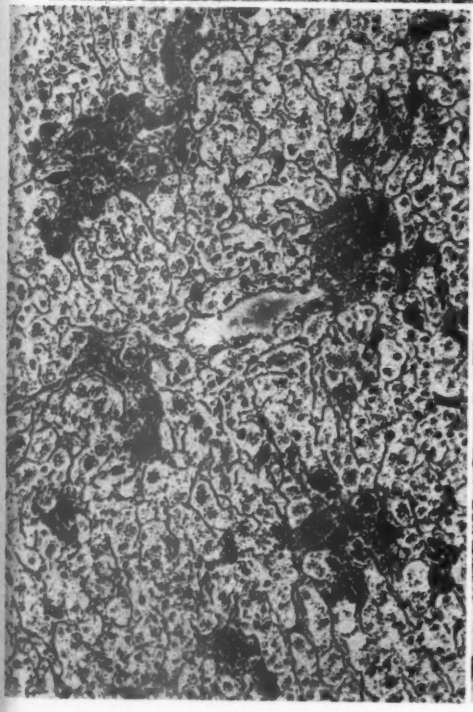

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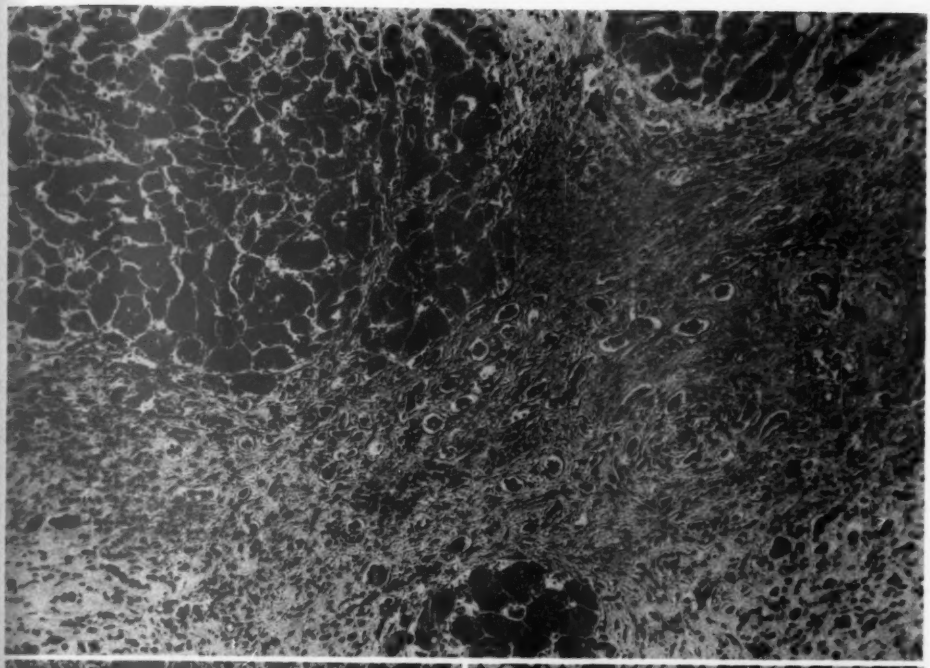
FIG. 6. Liver with severe cirrhosis, showing regenerating parenchymal nodules, some cells of which contain polyploid, bizarre nuclei. There is infiltration of the stroma by lymphocytes and histiocytes. Many newly formed bile ducts are present also. Hematoxylin and eosin stain. × 120.

FIG. 7. Liver with recent massive necrosis, showing collapse and degeneration of the parenchyma with condensation of the stroma in which there is mononuclear cell and lymphocytic infiltration. Many of the parenchymal cells are degenerated and bizarre. In addition, newly formed bile ducts are present. Hematoxylin and eosin stain. × 120.

FIG. 8. Liver from a child dying of kwashiorkor, showing marked fatty change and formation of "fatty cysts." The portal triad shows scanty cellular infiltration. Hematoxylin and eosin stain. × 120.



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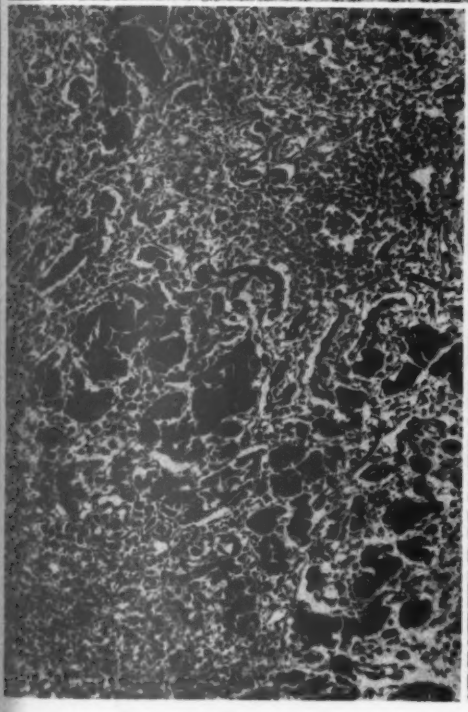

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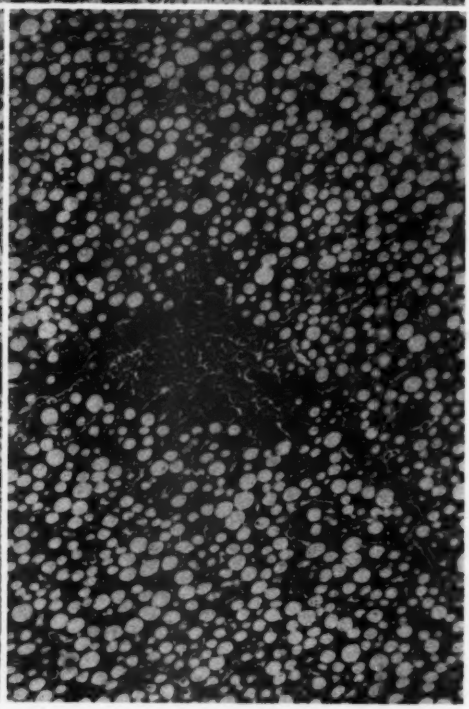

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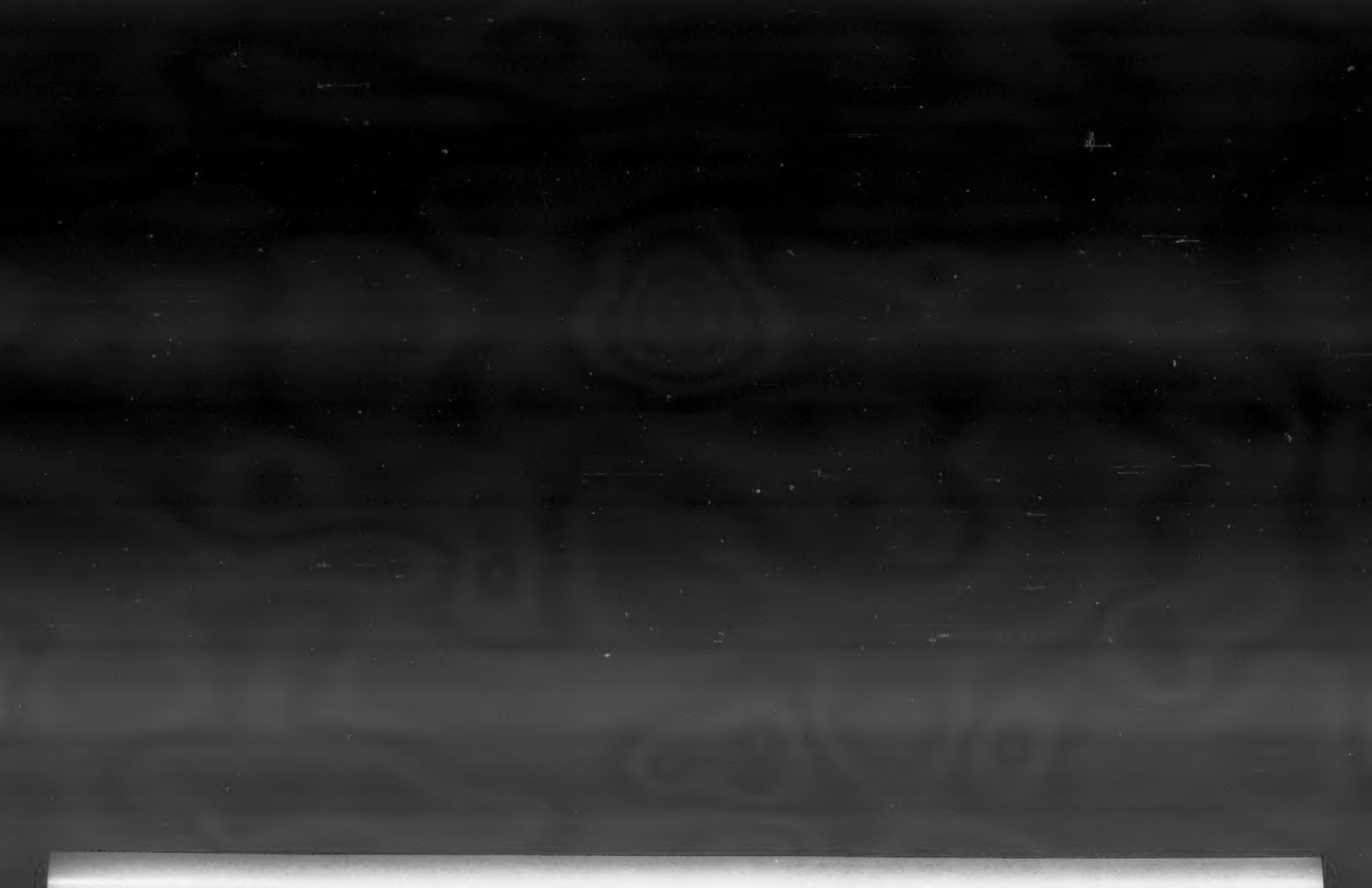
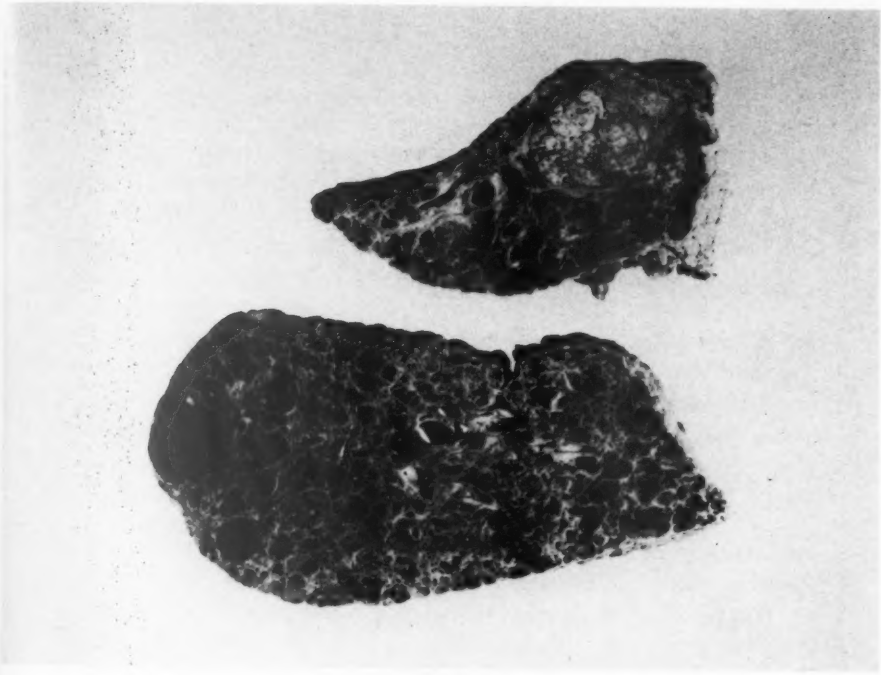
FIG. 9. Primary cancer arising in a liver with well marked nodular hyperplasia, probably of post-necrotic origin. Hyperplastic non-malignant nodules are present. × ½.

FIG. 10. Nodules of hepatocellular carcinoma arising in a liver with severe cirrhosis, probably of post-necrotic origin. Hematoxylin and eosin stain. × 30.





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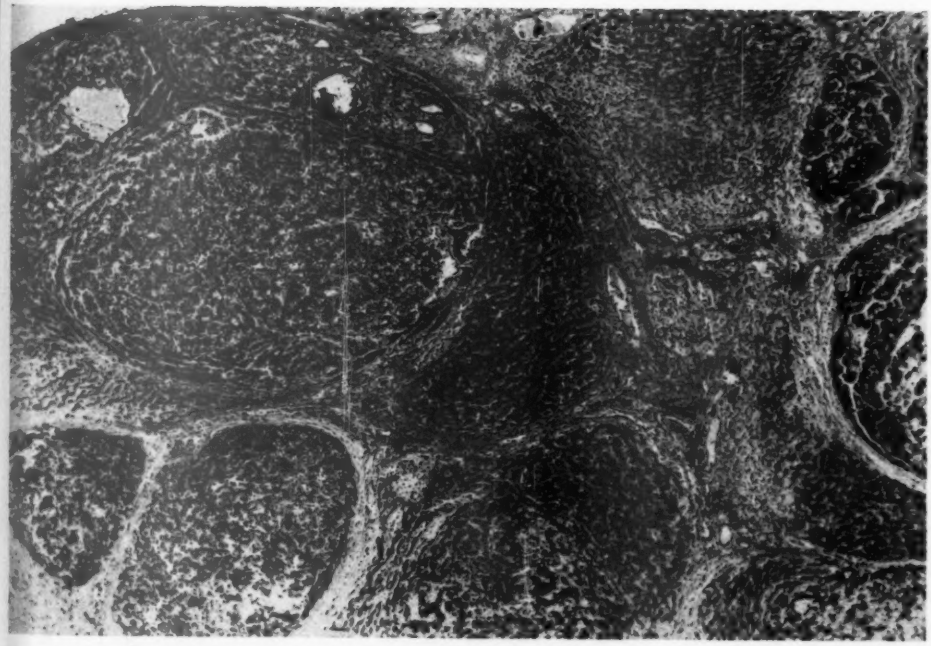

10

STUDIES ON THE NATURE OF FIBRINOID IN THE COLLAGEN DISEASES *

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Fibrinoid material is found in a great variety of human lesions from obvious inflammatory diseases, such as appendicitis, to afflictions of obscure etiology, such as those grouped under the generic term collagen diseases.[1-5] The nature of this fibrinoid material, however, is unknown; controversy continues over whether it represents degenerating collagen, altered ground substance, or simply fibrin deposited in the area.

In the study reported here, the evidence clearly indicates that, at the very least, fibrin is an integral component of such fibrinoid material. The fibrin is deposited in intimate association with collagenous fibers by the conversion of interstitial fibrinogen to fibrin *in situ*, presumably as the result of an inflammatory reaction in the area.

METHODS

Human Tissues. Specimens of human tissues were obtained at necropsy from the following diseases: rheumatoid arthritis (1 case), disseminated lupus erythematosus (4 cases), dermatomyositis (2 cases), subacute and chronic glomerulonephritis (3 cases), and periarteritis nodosa (1 case, kidney only). In addition, the following tissues had been obtained for biopsy: subcutaneous nodules from 8 and synovial membrane from one of 9 patients with rheumatoid arthritis, cardiac auricular appendages from 9 patients with rheumatic fever, and voluntary muscle from 2 patients with dermatomyositis. Tissue samples were studied also from 2 normal placentas and from 2 cases of acute fibrinous appendicitis.

Procedures. Each block of tissue was divided: one piece was frozen rapidly in bottles precooled to −70° C. and the other piece was fixed in Zenker's acetic acid fluid.

The unfixed frozen tissues were studied with Coons and Kaplan's[6] fluorescent antibody method. The tissues were sectioned at 4 μ at −30° C. in a special cryostat[6]; the sections were placed directly on

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slides and dried rapidly. The mounted sections were placed in 95 per cent alcohol for 30 seconds, washed for 20 minutes with four changes of 0.15 M NaCl, and then flooded with fluorescein-labelled[6] rabbit antiserum against human fibrin.[7-9] Rabbit antihuman fibrin antisera cross-react with human fibrinogen[7,8]; the washing procedure served to wash fibrinogen as well as other soluble proteins from the section, leaving behind the insoluble fibrin[9]; the alcohol was used simply to produce adhesion of the section to the slide and did not affect the subsequent solubility of fibrinogen in 0.15 M NaCl.

Replicate sections were stained with labelled antiserum that had been adsorbed either with purified fibrinogen or with washed fibrin prepared from purified fibrinogen solutions; these sections served as partial controls for the specificity of the staining reaction by removing specific antibodies. By pretreating the sections with unlabelled rabbit anti-fibrin and washing before flooding them with labelled antiserum, additional staining controls were obtained; in these instances, combination of the unlabelled antibodies with fibrin specifically inhibited the subsequent uptake of labelled antibodies. Sections were stained also with fluorescein-labelled rabbit antiserum against human serum albumin and against human γ-globulin. The latter sections were prepared in an attempt to obtain still further evidence for the specificity of the reactions of sections with anti-fibrin antisera.

All sections were washed following interaction with labelled antisera, using four changes of 0.15 M NaCl over a period of 20 to 30 minutes. The sections were mounted in buffered glycerol and examined with an ultraviolet light microscope for the presence of specific fluorescence indicating the distribution in the section of the protein being studied. Photomicrographs were taken of areas of specific fluorescence. The sections were then stained with Mallory's[10] phosphotungstic acid-hematoxylin (PTAH) or Biebrich scarlet[11] and the photographed areas re-examined with the visible light microscope.

By first fixing the frozen sections in alcohol followed by acetone and omitting the saline washing prior to interaction with labelled antisera, the distribution of fibrinogen, albumin, and γ-globulin was investigated. All fluorescein-labelled antisera used in this study were fractionated with alcohol,[7] treated twice with bovine liver powder,[6] and then twice with rabbit bone-marrow powder prior to use to remove conjugates that ordinarily add non-specific fluorescence to tissue sections.

Frozen sections cut in the cryostat were fixed also in Zenker's acetic acid and stained with Mallory's PTAH and a Biebrich scarlet-aniline blue sequence.

The duplicate tissue blocks fixed in Zenker's acetic acid fluid were embedded in paraffin, and 8 μ sections were stained with hematoxylin and eosin, Pearse's[12] periodic acid-leukofuchsin-orange G-hematoxylin sequence, a modification of McManus'[13] periodic acid-Schiff method (PAS), Mallory's PTAH, and the Biebrich scarlet-aniline blue method. It is to be emphasized that the interaction of these stains with fibrin is dependent upon the nature of the fibrin formed and that this in turn is dependent upon the composition of the medium in which the conversion of fibrinogen to fibrin occurs.[14] Thus negative staining reactions for fibrin with these non-specific dyes do *not* necessarily indicate the absence of fibrin from the tissue section.[14]

It must also be noted that the material in washed tissue sections reacting specifically with fluorescent anti-fibrin antibodies is considered to be fibrin, or an insoluble derivative of fibrinogen, since these are the only proteins in human tissues which will specifically interact with such antisera.[7-9] *Washed sections of normal human tissues do not reveal specific fluorescence after interaction with labelled anti-human fibrin antisera and hence do not contain free fibrin or immunologically reactive insoluble derivatives of fibrinogen; soluble* fibrinogen is a normal component of interstitial fluids and thus is readily demonstrable in *unwashed* tissue sections.

Results

It became evident very early in the study that the distribution of fibrin, as determined by the specific antibody method, was usually more extensive than was indicated by the standard histologic methods. In many instances, fibrin revealed by specific fluorescence was partially or entirely negative for fibrin with the dyes. On the other hand, if dye-staining revealed fibrin in the fixed, paraffin-embedded sections, the specific antibody method regularly confirmed its presence.

Appendicitis. In the two inflamed appendices studied, a deposit of fibrin was obvious over the serosal surfaces with all of the stains employed (Fig. 1). Fibrinoid material was demonstrated in the submucosa using the dye methods and, in these areas, fibrin was found to be present using the fluorescent antibody technique; the fibrin was in masses and as fibrils intimately intermingled with the collagenous fibers (Fig. 2). Small masses of fibrin were found also in the intercellular spaces of lymphatic follicles.

Normal Placenta. Fibrinoid material, as has been described by others, was found in normal placentas. The fibrinoid material reacted as fibrin when studied with fluorescent antibodies. Fibrin masses, present on the villous surfaces, were apparent with both the fluorescent

and dye-staining methods, but in other areas, particularly within the substance of the villi, the fluorescent antibody method revealed fibrin which could not be detected by standard histologic techniques. In some areas, fibrin strands and collagen fibers were intermingled so closely that the two were indistinguishable morphologically when using dyes (Figs. 3 and 28); in some places, these areas were continuous with obvious masses of solid fibrin surrounding chorionic villi.

Rheumatoid Arthritis. All of the 8 subcutaneous nodules examined revealed the typical picture of the nodules of rheumatoid arthritis. Masses of swollen fibrillar collagen could be found arranged about single or multiple necrotic centers. The latter were bordered by typical palisading fibroblasts. At the periphery of such edematous collagen, the transition to the compact, denser, collagenous bundles of more normal subcutaneous tissue was quite abrupt. Characteristically, some portion of the whorls formed by the edematous and swollen collagen contained fibrin by the specific antibody method, either as large masses or as a fine reticular pattern gradually intermingling or merging with the fine fibers of collagen in the areas; this fibrin usually was found either in the central areas formed by the whorl or at the periphery where the convolutions of fibrous tissue came together (Figs. 4 to 8 and 29 to 32). The dye methods indicated these central portions to be areas with fibrinoid material, and it was noted that (1) frequently only portions of the fibrin demonstrated by specific antibodies were positive for fibrin with the dyes used and that (2) the fine reticular fibrils of fibrin often could not be distinguished by dyes from the reticular collagen fibers with which they intermingled. Positive staining of part of the fibrin fibrils with dyes made it appear as though edematously separated fibers of collagen had suddenly developed the altered staining reaction of fibrinoid (Fig. 32); the specific antibody method readily revealed these fibrin strands intermingling intimately with the collagen fibers.

In some instances, remnants of dense collagen were found as islands in the looser, reticular collagen close to the centers of the whorls (Figs. 7 and 8). On several occasions, fibrin was discovered in the matrix of these dense collagenous masses (Figs. 8 and 31).

The usual cellular components of subcutaneous nodules were present, but it is important to note that the areas of greater round-cell concentration were usually at the periphery of the palisading fibroblasts surrounding the fibrin masses.

At necropsy, the patient who succumbed to rheumatoid arthritis had severe pericarditis and fibrinous pleurisy. Her arthritic symptoms had ceased some time prior to death. The heart, lungs, and spleen re-

vealed an acute fibrinous serositis. No fibrinoid degeneration was detected in any organs of this patient by dye methods, although fibrin was readily demonstrable on the visceral pleural (Fig. 9) and pericardial surfaces. No "degenerative" lesions were found in the myocardium or endocardium, but intimal proliferation was seen in a coronary artery, and fibrin was present abundantly in the outer half of the intima in such areas (Fig. 10). An interesting finding was the presence of fragments of fibrin in the reaction centers of the malpighian follicles of the spleen (Fig. 11); the eosinophilic masses seen in such reaction centers, in the variety of conditions studied here, appeared to be fibrin.

Biopsy of the synovial membrane of the patient with rheumatoid arthritis showed lymphocytic and mononuclear foci, loose fibrous tissue, and granulation tissue consistent with rheumatoid arthritis. Scattered throughout the loose reticular fibrous tissue were intermingling fibrils and small foci of fibrin (Fig. 12). Fibrin was found on the synovial surface as well, by both the dye methods and by fluorescent antibodies. It should be noted that this patient also had agammaglobulinemia and no γ-globulin could be detected in the specimens of synovium, skin, or muscle that were obtained for biopsy.[8]

Rheumatic Fever. Fully developed active Aschoff bodies were not found in any of the necropsy material available during the course of the study. Some lesions, however, were found in three of nine auricular appendages obtained during valvulotomy. In these instances, some small endocardial lesions were present. Only thickened intima with some organization was indicated by the dyes in these areas, but the fluorescent antibody method revealed small masses and fibrils of fibrin at the centers of these lesions (Fig. 13).

Disseminated Lupus Erythematosus. In the cases studied, the classical picture of disseminated lupus erythematosus prevailed, with involvement of heart, kidneys, spleen, and skin. The myocardium showed widespread involvement of muscle fibers with focal areas of necrosis. The fluorescent antibody method invariably revealed fibrin surrounding the involved muscle with the frequent occurrence of fibrin in focal areas of "fibrinoid degeneration" in the myocardial connective tissue (Figs. 14 and 15). Fibrils and small masses of fibrin intermingled with loose collagen could be found in the verrucal "pocket" lesions of the mitral valve (Fig. 16). Blood vessels in the myocardium frequently showed fibrinoid changes in the media (Fig. 33): the fibrinoid material was found to be fibrin (Fig. 17) and fibrin could be demonstrated also in the thickened intima.

In the spleen, fibrin was abundant in the malpighian follicles, almost

filling the interstitial space (Figs. 18 and 19) and often outlining the small central arterioles. Fibrin could be found also in small amounts in the media of the central arterioles, accentuating the typical "onion-skin" appearance (Fig. 19).

In the kidney, fibrin was found in the glomerular tufts, representing the fibrinoid material in these (Fig. 20), but crescents and fibrin deposition in Bowman's spaces were unusual. In these glomeruli, Mallory's PTAH stain and the Biebrich scarlet method were negative for fibrin, but MacManus' PAS stain was intensely positive in regions similar to the fluorescing areas. In the kidneys from another child, the wire-loop appearance of the glomerular tufts so typical of this disease was very well developed. Using the dye methods, abundant fibrinoid material was present in the thickened capillary walls of the tufts (Fig. 24). The fluorescent antibody method showed these walls to be heavily impregnated with fibrin (Fig. 25).

In the lungs of one child the alveoli were filled with fibrin masses with some membrane formation. In another child, fibrinoid changes in dermal collagen were found and the fibrinoid appeared to be composed of fibrin.

Dermatomyositis. In all tissues obtained at necropsy and for biopsy from patients with dermatomyositis, fibrinoid degeneration was conspicuously absent when sections were studied by dye-staining. The fluorescent antibody method revealed fibrin amidst the inflammatory cells infiltrating the connective tissues of skin and muscle and, occasionally, fibrin strands in edematous connective tissue at the periphery of the cellular infiltrates.

Subacute and Chronic Glomerulonephritis. In one child with subacute glomerulonephritis, fibrin deposition and crescent formation in Bowman's space were very conspicuous. In the crescents, fibrin was shown to be present by the fluorescent antibody method; fibrin was found also in the glomerular tufts (Fig. 21).

In 2 cases of severe chronic glomerulonephritis, despite marked hyalinization, an occasional hyalinized glomerular tuft contained fragments of fibrin. However, some glomeruli, not so extensively hyalinized, showed more numerous flecks of fibrin (Fig. 22). Fibrin was not conspicuous in these kidney sections as a rule. Incidentally, one child succumbed with uremic pneumonitis and the lungs showed typical hyaline membranes and hyaline masses filling some of the alveoli. The hyaline membranes and the masses have been shown to be composed principally of fibrin,[9] using the specific antibody method (Figs. 23 and 34).

Periarteritis. In the kidney specimen obtained from a patient with periarteritis, almost all arteries and arterioles were involved. Organization of old thrombi and intimal proliferation were present in many blood vessels, often almost to the point of complete occlusion of the lumen, and in these vessels the internal elastic lamina was often discontinuous in places (Fig. 26). Abundant coarse trabeculae of fibrin and delicate fibrin strands were found in such areas of intimal proliferation, mixed with the granulation tissue and forming an appreciable portion of the bulk of the organizing material, particularly toward the luminal areas. In some cases, no fibrinoid change was demonstrable by hematoxylin and eosin stains, but where fibrin was found by the fluorescent method there was always evidence of previous damage in the area. In other blood vessels, the entire wall was necrotic (Figs. 27 and 35) with loss of both elastic membranes. The great mass of hyaline material replacing the vessel wall was found to be composed, in part at least, of fibrin as determined by specific fluorescence. Some portions of the fibrin in the necrotic vessel walls did give a positive reaction for fibrin with Pearse's staining method and Biebrich scarlet, but most of it did not (Figs. 36 and 37).

Discussion

The rabbit antisera against human fibrin that were used in this study did not contain antibodies against any proteins in human serum and have been shown to be specific for fibrin and fibrinogen.[7-9] The evidence presented indicates that areas containing fibrinoid material found in a variety of human lesions, particularly those associated with "collagen diseases," reacted specifically with these antisera. It would seem, therefore, that fibrinoid is, at least in part, an insoluble derivative of fibrinogen, viz., fibrin.

It is conceivable, however, that insoluble derivatives of fibrinogen other than fibrin may exist in these lesions. It has been suggested, for example, that certain mucoitin polysulfuric esters may precipitate fibrinogen *in vivo* in the pathogenesis of the generalized Shwartzman reaction.[15,16] Such precipitates produced with available mucoitin sulfates ordinarily are not soluble *in vitro* at 37° C., and thus it is admittedly possible that local injury might release certain mucoitin sulfates from connective tissue ground substance, which would have the capacity to precipitate fibrinogen at body temperatures. Evidence for this, however, is lacking. A positive PAS staining of fibrinoid does not necessarily indicate the presence of such polysaccharides, since fibrin alone gives a positive PAS reaction. In addition, it would be necessary

to postulate that the interaction of such mucoitin sulfates and fibrinogen is capable of producing fibrils without conversion of the fibrinogen in the complex to fibrin, since most of the material in the lesions studied was fibrillar. It is possible that, during formation of the lesions, certain reactive groups of the collagen fibers are exposed which would bind a thin layer of fibrinogen specifically and tightly enough to resist removal by 0.15 M NaCl. Against this possibility is the fact that the fibrin in these lesions was present as masses or as fibrillar strands occupying spaces between fibers rather than on the collagenous fibers.

The conclusion that fibrinoid contains fibrin does not preclude the presence of other substances in the bulk of fibrinoid as seen in a lesion stained by standard histologic procedures. The plasma proteins, for example, which are normally present in the interstitial fluid of all tissues, may increase in concentration in areas of inflammation. Necrotic tissues, particularly if cellular elements such as inflammatory cells are involved, undoubtedly contribute enzymes, thromboplastic substances, nucleoproteins, and other materials to these lesions. Since fibrinogen is a plasma protein normally present in interstitial fluid, the presence of fibrin in the lesions is not at all surprising.

The evidence suggests that fibrin deposition as fibrinoid is not restricted to the collagen diseases, but is found in other inflammatory conditions as well. The presence of fibrin in the lesions studied indicates simply that there has been a local conversion of fibrinogen to fibrin. For this conversion to occur, however, an abnormal release of thromboplastic substances into the interstitial fluid of the area must take place; these substances are undoubtedly contributed by local tissue deterioration or necrosis. The large masses of fibrin found and the edema in these lesions strongly suggest that a local increase in vascular permeability is part of the pathologic process, resulting, among other things, in an increase in fibrinogen in the area. Actually, these processes are to be expected in any area of tissue destruction. This study, therefore, gives no indication as to the nature of the etiologic agents involved in the collagen diseases, since it is apparent that tissue destruction may result from such diverse causes as viral infection, bacterial toxins, or hypersensitivity.

It was noted that the fluorescent-antibody method was much more sensitive for the detection of fibrin in tissue sections than were the more conventional histologic stains and that, in many instances, the latter gave negative results for fibrin where it was apparent in abundance with the former technique. It has been shown that the interaction of fibrin with dyes is dependent upon the medium in which the

conversion of fibrinogen to fibrin occurs.[14] In the absence of a protein component referred to as the Laki-Lorand factor,[17] or of relatively large amounts of glutathione, albumin is necessary if the resulting fibrin is to stain as does a plasma clot.[14] It would seem from available information that the albumin concentration in interstitial fluid, which is normally about 0.7 to 1.0 gm. per cent, is barely sufficient for the production of the form of fibrin which would stain positively for fibrin with the usual dyes. The concentration of interstitial albumin, however, may be increased locally by inflammatory processes. Consequently it is necessary to emphasize that the biochemical parameters for the production of the dye-positive form of fibrin are not yet clearly delineated.

Summary

Sections of tissues from patients with rheumatoid arthritis, disseminated lupus erythematosus, dermatomyositis, rheumatic fever, subacute and chronic glomerulonephritis, and periarteritis nodosa were studied with fluorescein-labelled rabbit antisera against human fibrin, albumin, and γ-globulin. Suitable controls were used to insure the specificity of the method for the detection of these proteins in sections of human tissues. Replicate sections were studied with phosphotungstic acid-hematoxylin, Biebrich scarlet-aniline blue, periodic acid-leukofuchsin-orange G-hematoxylin, and periodic acid-leukofuchsin; these methods ordinarily are considered suitable for the detection of fibrin. Tissue samples from normal placentas and from acutely inflamed appendices were studied also.

The data obtained indicate that the fibrinoid material found in the lesions associated with the collagen diseases is, at least in part, fibrin. It was observed also that the conventional staining methods employed were not reliable for revealing fibrin in tissue sections, since they frequently gave negative results for areas in which fibrin could be demonstrated with specific fluorescent antibodies. The significance of these findings is discussed.

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LEGENDS FOR FIGURES

Figs. 1 to 23 and Figs. 25 to 27 are photomicrographs of washed tissue sections treated with fluorescein-labelled rabbit anti-human fibrin as described in the text. The white areas represent specific fluorescence due to interaction and fixation of labelled anti-fibrin antibodies with antigen in the sections. The grayish areas are the result of a bluish autofluorescence of certain tissue structures which, under the ultraviolet microscope, is easily distinguished from the greenish yellow fluorescence of the fluorescein label. All photomicrographs of Figures 1 to 27 were enlarged 4 times their original size.

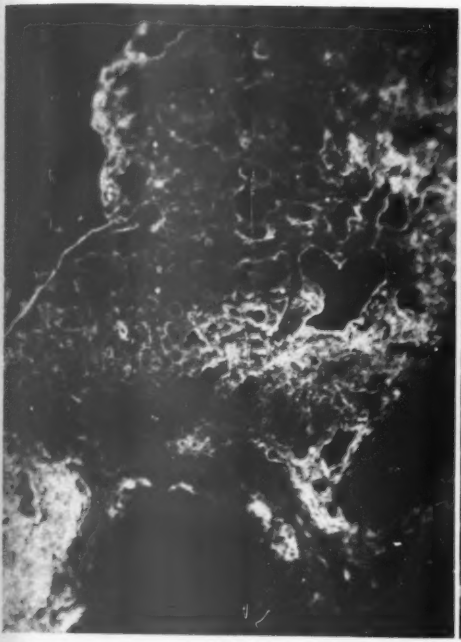
FIG. 1. Fibrin present on the serosal surface of an inflamed appendix. × 100.

FIG. 2. Fibrinoid reacting for fibrin in the submucosa of the same section as Figure 1. × 210.

FIG. 3. Section of normal human placenta. Arrows point to areas of fibrinoid in which fibrin strands and collagen fibers are intimately intermingled; for comparison with Figure 28. × 100.

Figs. 4 to 8. Sections of different rheumatoid subcutaneous nodules showing areas of fibrinoid composed of fibrin strands and masses.

FIG. 4. Diffuse deposition of fibrin strands intermingling with collagen at the periphery. For comparison with Figure 29. × 210.



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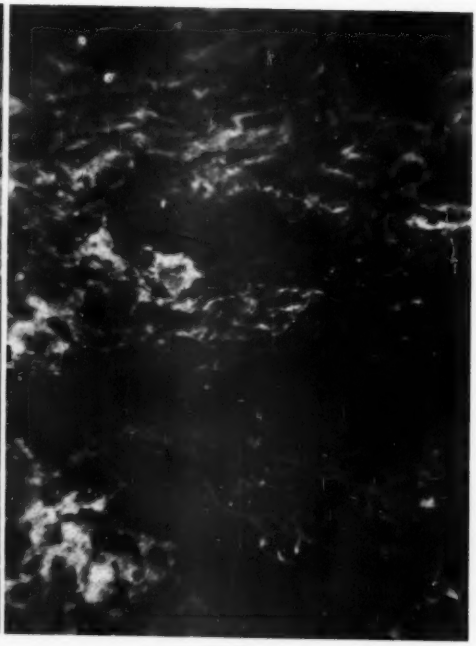

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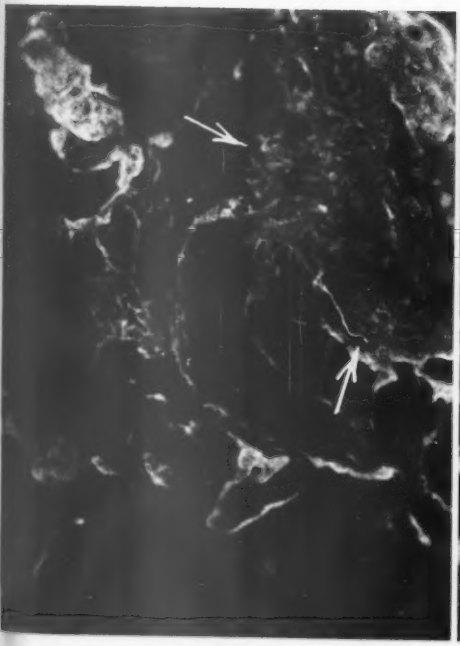

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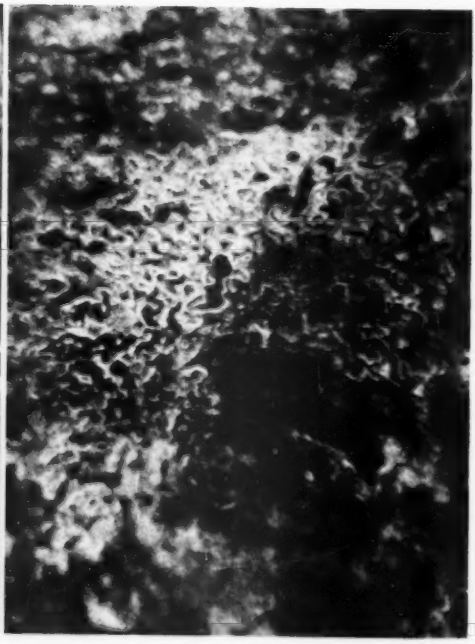

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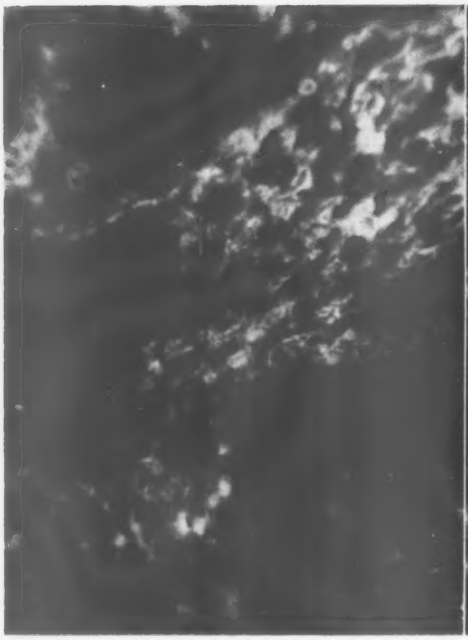

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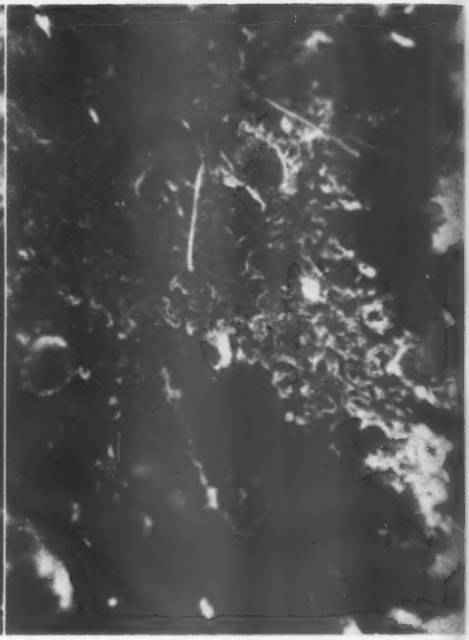

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FIG. 5. Coarse fibrin which was only partly distinguishable from collagen using dye methods (Fig. 32). × 210.

FIG. 6. Necrotic center; round gray masses are older, dense, collagen bundles among swollen, reticular, collagen fibers and within the necrotic center; for comparison with Figure 30. × 210.

FIG. 7. Flecks of fibrin in the center of loose whorling collagen fibers close to and occasionally (arrow) within dense collagen masses. × 210.

FIG. 8. The necrotic center of a whorl of reticular collagen showing not only fibrin in the necrotic area but also in some of the dense collagen masses (arrows); for comparison with Figure 31. × 100.

FIG. 9. Fibrin (upper right-hand portion) overlying visceral pleura of lung in patient with rheumatoid arthritis. × 210.

FIG. 10. Fibrin in the thickened intima of a coronary artery in a patient with rheumatoid arthritis. Arrows indicate fragmentation of internal elastic membrane which is revealed by intense blue autofluorescence. × 210.

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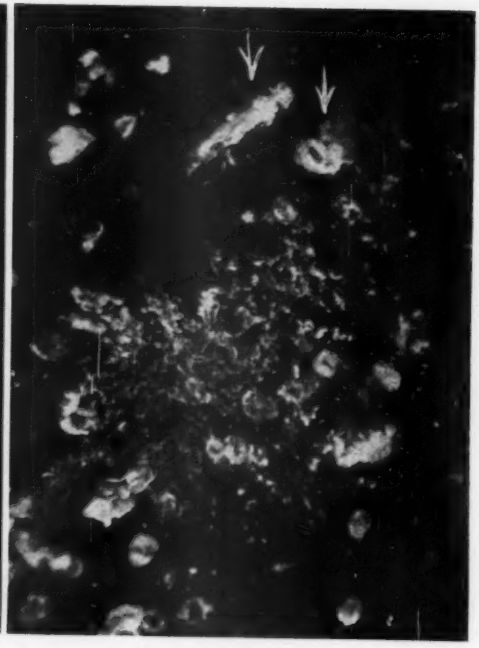

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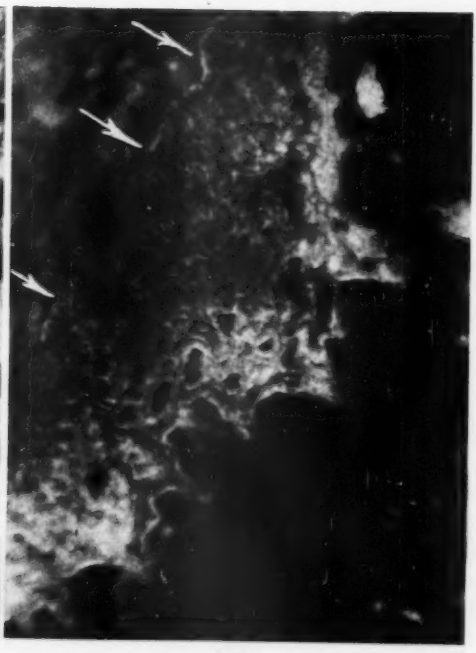

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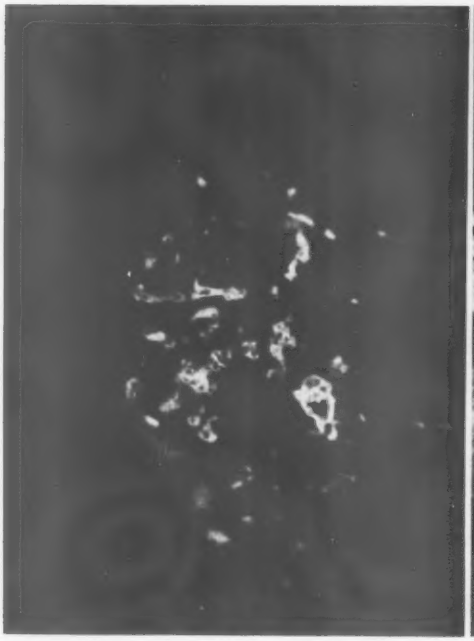

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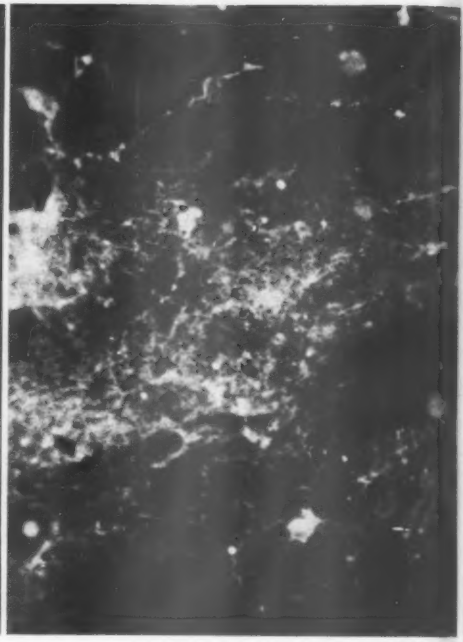

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FIG. 11. Fibrin masses in reaction center of malpighian follicle of spleen in patient with rheumatoid arthritis. × 100.

FIG. 12. Fibrin masses and strands in synovium of patient with rheumatoid arthritis and agammaglobulinemia. × 100.

FIG. 13. Fibrin in an area of subsiding activity in left auricle of patient with rheumatic fever. × 210.

FIG. 14. Fibrin deposited in and around necrotic muscle fibers in the myocardium from a case of lupus erythematosus. × 210.

FIG. 15. Fibrin strands intermingled with collagen in interstitial connective tissue of myocardium in the same section as that shown in Figure 14. × 210.

FIG. 16. Fibrin strands in area staining as fibrinoid in verrucal pocket lesion in lupus erythematosus. × 210.

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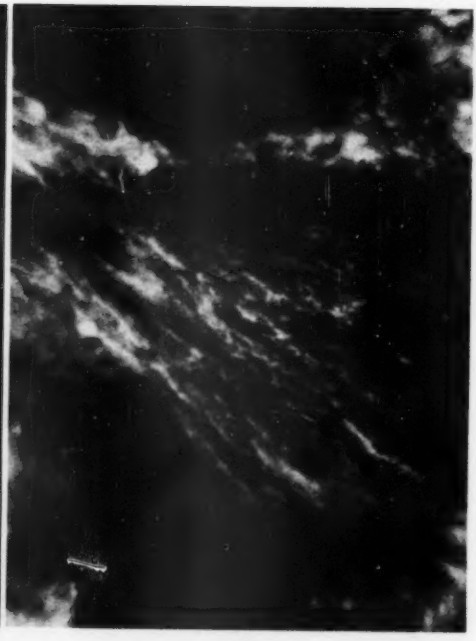

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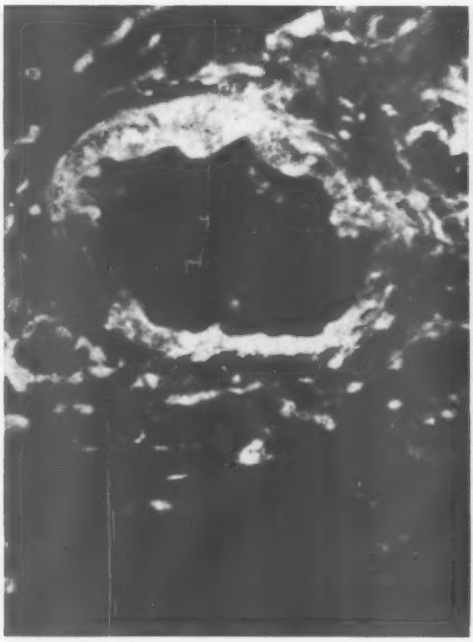

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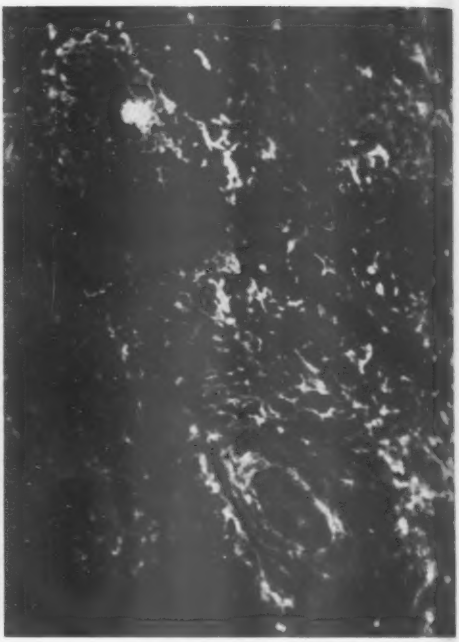

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FIG. 17. Fibrin deposition in wall of coronary vessel in patient with lupus erythematosus, staining as fibrinoid with the dye methods; for comparison with Figure 33. × 100.

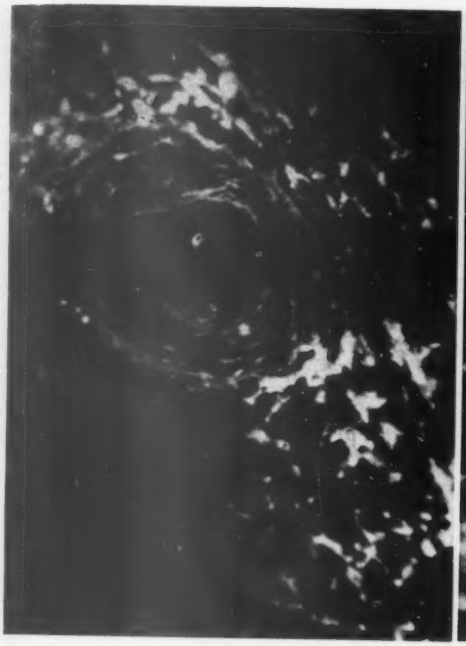
FIG. 18. Fibrin masses in reaction center of splenic follicle in lupus erythematosus. × 100.

FIG. 19. Lamellar deposition of fibrin in penicillar vessel of splenic follicle in the same section as that shown in Figure 18. × 210.

FIG. 20. Fibrin in renal glomerular tuft in patient with lupus erythematosus. × 210.

FIG. 21. Fibrin in glomerular tuft (upper portion of photograph) and in a glomerular crescent (lower portion) of kidney from patient with subacute glomerulonephritis. × 210.

FIG. 22. Fibrin in an unhyalinized glomerular tuft of kidney from patient with chronic glomerulonephritis. × 210.



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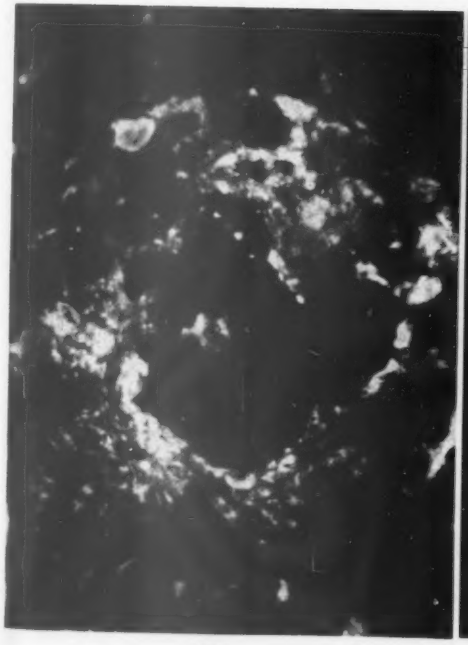

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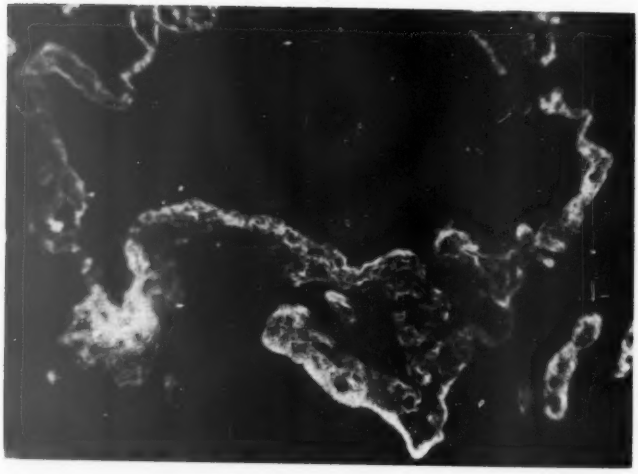
FIG. 23. Fibrin composing a hyaline membrane in lung from a patient who succumbed in uremia; for comparison with Figure 34. × 210.

FIG. 24. Section of kidney from patient with lupus erythematosus, stained with hematoxylin and eosin. Glomerular tufts have abundant fibrinoid material in the swollen capillary walls. × 100.



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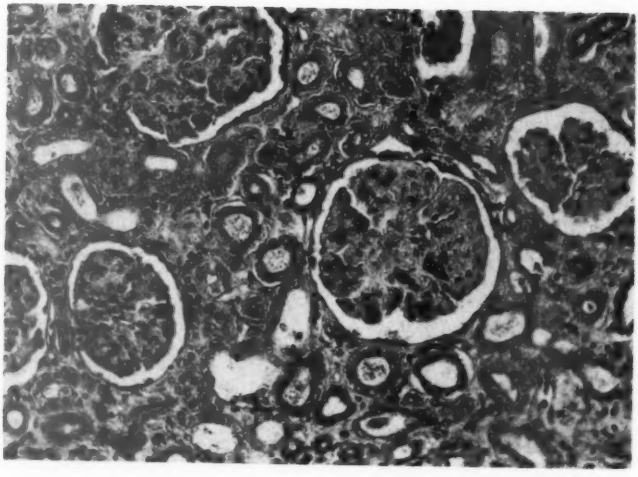

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FIG. 25. Fibrin in a section from the same kidney as shown in Figure 24. The fibrin in the capillary walls of the glomerular tuft is apparent. × 210.

FIG. 26. Fibrin in proliferating intima of a small renal artery from patient with polyarteritis nodosa. Arrows indicate fragmented internal elastic membrane seen by autofluorescence. × 210.

FIG. 27. Fibrin in the necrotic wall of another small renal artery in same section as shown in Figure 26; for comparison with Figures 35, 36, and 37. × 210.

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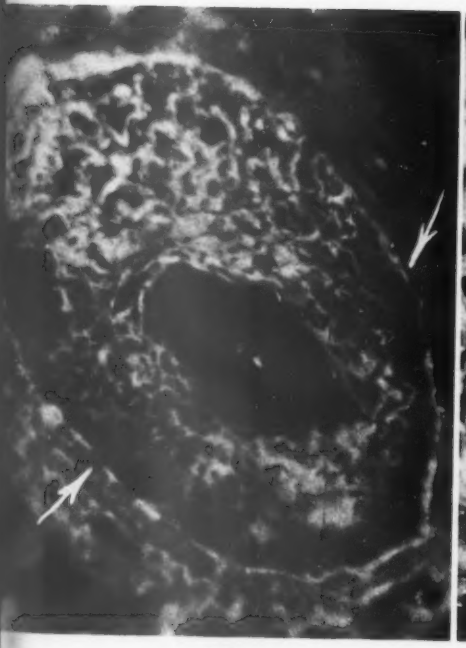

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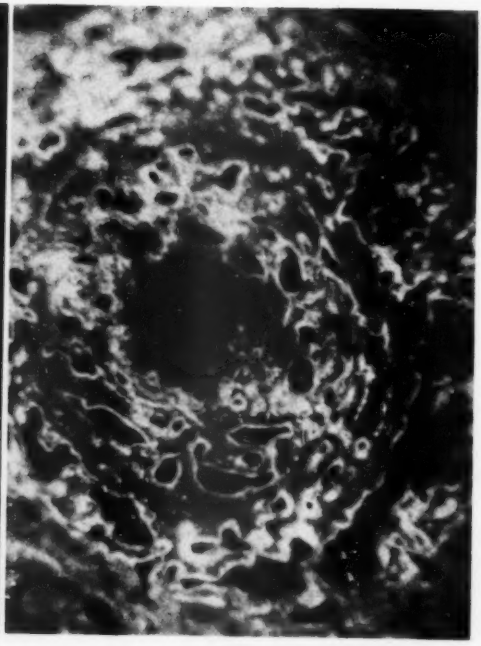

27

Figs. 28, 29, 31, 34, and 35 are photomicrographs of sections that were cut at −30° C., examined for fibrin by the fluorescent antibody method, and then restained. Figures 30, 32, 33, 36, and 37 are sections from tissues fixed in Zenker's acetic acid fluid and stained for fibrin with dyes.

FIG. 28. Normal placenta of Figure 3, restained with Biebrich scarlet-aniline blue. × 100.

FIG. 29. Same section of subcutaneous nodule as that shown in Figure 4, restained with PTAH. × 200.

FIG. 30. Fixed section, a replicate of Figure 6, stained with Biebrich scarlet-aniline blue. × 200.

FIG. 31. Same section of rheumatoid nodule as that shown in Figure 8, restained with Biebrich scarlet-aniline blue. × 200.

FIG. 32. Rheumatoid subcutaneous nodule stained with PTAH showing that only portions of the fibrin strands present are actually positive for fibrin with this method (arrows). × 200.

FIG. 33. Fixed section, replica of that shown in Figure 17, stained with hematoxylin and eosin and showing fibrinoid material in the media of a coronary vessel in lupus erythematosus. × 200.

FIG. 34. Same section as that shown in Figure 23, restained with Biebrich scarlet-aniline blue. × 180.

FIG. 35. Same section as that shown in Figure 27 restained with PTAH. × 180.

FIGS. 36 and 37. Fixed sections of small renal arteries from a patient with periarteritis nodosa, replicas of the section of Figure 27, stained with PAS and Biebrich scarlet-aniline blue, respectively. × 100.

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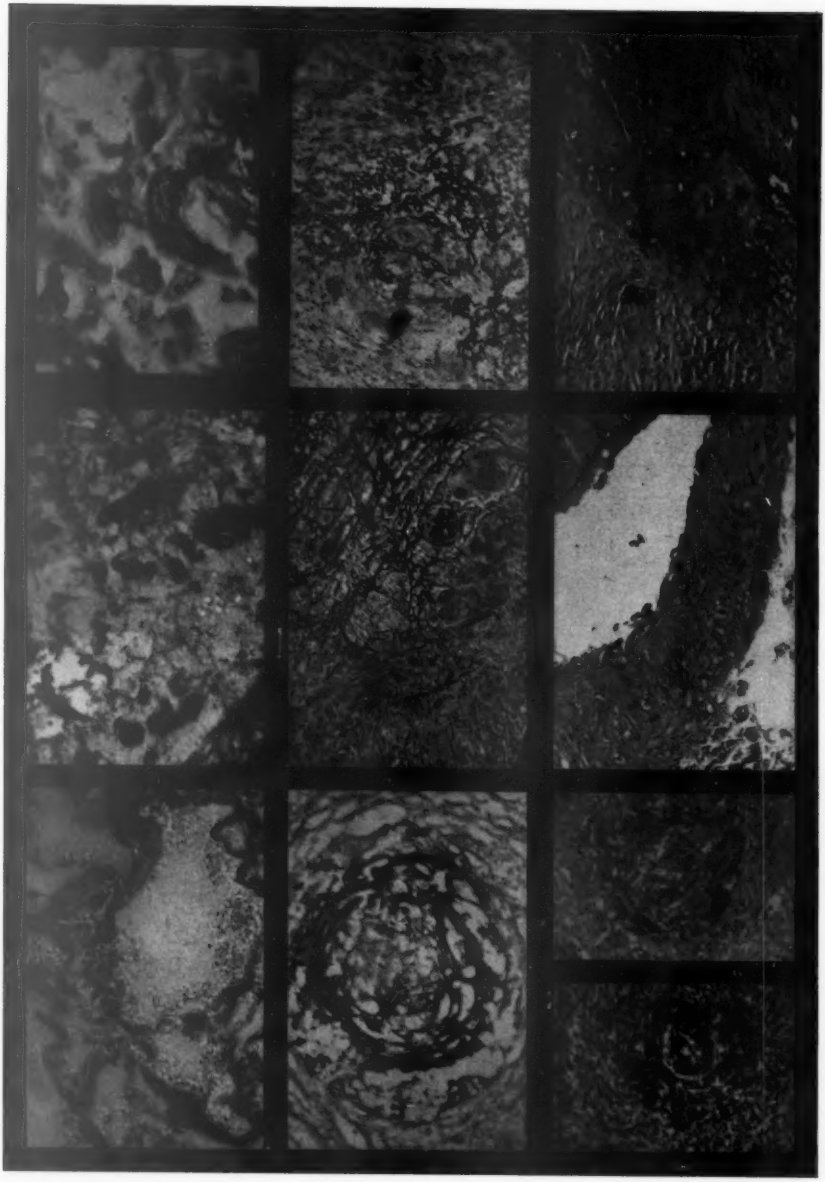

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STUDIES ON TRICHINELLA SPIRALIS

VI. Effects of Cobalt-60 and X-Ray on Morphology and Reproduction *

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From the Atomic Energy Commission Laboratory on Biological Effects of Irradiation, the Michigan Memorial-Phoenix Project, and the Department of Pathology, University of Michigan, Ann Arbor, Mich.; and the Departments of Pathology, Wayne County General Hospital, Eloise, Mich., and Wayne University College of Medicine, Detroit, Mich.

In previous studies with *Trichinella spiralis*[1,2] it was observed that when larvae were exposed to various amounts of gamma radiation, sexual sterilization of adults or inhibition of maturation was produced by x-ray in doses that were decidedly lower than those of cobalt-60. Sexual sterilization of adult trichinae followed exposure of larvae to doses of 3,500 to 5,000 r. (roentgens) x-ray or to a dose of 12,800 rep (roentgen equivalent physical) cobalt-60. Inhibition of maturation of larvae to adult worms was produced by 5,000 to 6,000 r. x-ray or by 18,000 rep cobalt-60. In the present communication, we submit data on the amounts of radiation with x-ray and cobalt-60 that produce morphologic changes in intestinal trichinae, prevent their reproduction, and cause their early elimination from the intestine of the host.

In 1916 Tyzzer and Honeij[3] applied radium to encysted trichinae thereby rendering the larvae non-infective to mice. Schwartz[4] in 1921, fed rats trichinous pork that had been exposed to x-ray. He found that x-ray had a selective and destructive effect on the gonads of adult trichinae, "relatively large doses" of x-ray preventing maturation of larvae to adult worms, and somewhat smaller doses permitting larvae to attain structural but not sexual maturity. Semrad[5] (1937) observed, in feeding experiments, that isolated larvae, exposed to 1,200 r. or more of x-ray, developed to adult forms but the adult females did not produce young. Evans, Levin, and Sulkin[6] (1941) determined that after irradiating larvae with 400 r. x-ray, the mean number of worm-shaped embryos that developed in the adult females was appreciably diminished, and that with 2,000 and 2,500 r. no embryos were found, although many late cleavage forms were present. Doses of 3,000 to 3,750 r. stopped embryonic development in early cleavage, and a dose

* These studies were carried out (1) as part of Project No. 54 of the Michigan Memorial-Phoenix Project; and (2) under Contract No. AT (11-1)-75 for the United States Atomic Energy Commission at the University of Michigan; and (3) with the assistance of the William J. Seymour Research Fund, Wayne County General Hospital.

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of 5,000 r. inhibited development of the irradiated larvae. Our own observations are in fairly close agreement with the findings of Evans, Levin, and Sulkin.

Alicata[7] (1951) found that higher doses of x-ray were necessary to produce sexual sterilization (9,000 to 10,000 r.) or to prevent maturation of larvae to adults (15,000 to 20,000 r.). After exposing larvae to various doses of x-radiation ranging from 4,000 to 10,000 r., he observed that the ovary of the adult females was shrunken and malformed; that the adult males had little evidence of degeneration but a great reduction in the number of sperm cells in the seminal vesicle; that the intestinal trichinae were stunted; and that the cuticle of the adult forms, especially females, was wrinkled and had abnormal thickenings along the distal half of the body.

Method

White rats weighing approximately 175 gm. were each infected with 5,000 non-irradiated Trichinella larvae (controls) or with an equal number of larvae that were exposed to doses of cobalt-60 ranging from 1,000 to 30,000 rep or to doses of x-ray ranging from 1,000 to 8,000 r. The larvae were suspended in tap water in lusteroid test tubes and exposed to cobalt-60 from a kilocurie source having a radiation rate of 1,333 rep per minute (as measured with ferrous sulfate dosimeters, using the method of Weiss[8]), or to x-ray from a machine having a tube with tungsten anode, operated at 245 kv., 18 ma., with filtration of 0.5 mm. copper and 1 mm. aluminum. A Victoreen rate meter was used to indicate the x-ray output. The distance from the target of the x-ray tube to specimen being irradiated was 23 cm. and the dose rate was 312 r. per minute. For the range of energies employed and the types of material irradiated, it has been assumed that the terms roentgen unit (r.) and roentgen equivalent physical unit (rep), as measured, may be used interchangeably.

After removal from the body, the small intestine was placed in a petri dish and cut into six segments, each about 5 cm. long. These were opened longitudinally. The contents of the petri dish were transferred to a quart jar which was then filled with tap water, shaken vigorously, and placed in the refrigerator (at 7° C.) overnight. The following morning the segments of intestine were transferred to a second quart jar which was vigorously shaken, and the pieces of bowel were removed and discarded. Microscopic examination of the supernatant fluids that were to be discarded revealed in each sample some nonmotile trichinae, whereas motile trichinae settled to the bottom of the suspension. Substitution of saline solution for tap water as the medium

of suspension permitted nearly all the trichinae to remain motile. Accordingly, in subsequent experiments (on rats fed larvae irradiated with 5,000 rep or more cobalt-60, and all rats fed larvae exposed to x-ray) the small intestine was washed with physiologic saline solution rather than with tap water, thus permitting a larger number of trichinae to be recovered. The sediment from both jars was concentrated to a volume of 50 to 100 ml. and repeatedly washed until the supernatant became clear. Counts were then made of the number of trichinae in samples of the final sediment.

Microscopic sections of tissue from the small intestine were examined for trichinae. In animals sacrificed 6 days or more after infection, blocks of tissue were taken also from the heart for evidence of focal granulomatous inflammation induced by early invasion of larvae.

As a test of the ability of the worms to reproduce in the intestines of rats, groups of control and test rats were sacrificed approximately 35 days after infection and tissue for microscopic sections was taken from the tongue, diaphragm, skeletal muscle, and heart for presence and degree of trichinous infection or for evidence of inflammation. The skinned and eviscerated carcass was ground in a Waring blendor and digested in an incubator, in an aqueous solution containing 1 per cent pepsin and 1 per cent hydrochloric acid, for recovery of larvae of the second generation.

The number of trichinae persisting in the small intestine was determined at 3 and 6 days, and in some instances at 2 to 48 hours, after infection of rats with larvae exposed to various doses of radiation.

Trichinae to be measured were freed from the intestine and washed several times in physiologic saline solution. They were then fixed in a solution of hot formalin. Worms to be stained were fixed in a mixture consisting of 95 ml. of 70 per cent hot alcohol and 5 ml. of glycerin. This fixative appeared to be preferable to formalin, especially for preparing permanent mounts. The worms were stained with alum cochineal and counterstained with fast green. With an ocular micrometer measurements were made of the length and maximum width of the body and of the gonad of 10 male and 10 female trichinellae at 3 and 6 days and, in a few instances, at 24 to 48 hours after worms had developed from non-irradiated larvae (controls) or from larvae exposed to various doses of cobalt-60 or x-ray. In each experiment, one portion of excysted larvae was exposed to a given dose of radiation and fed to test rats, while another portion of the same lot of excysted non-irradiated larvae was fed to control rats.

Direct observations were made at various intervals, up to 144 hours after infection, on the structure of living worms that developed from

non-irradiated larvae and from larvae exposed to x-ray (2,000 to 6,000 r.) or to cobalt-60 (1,000 to 18,000 rep). Microscopic observations, both on living and fixed trichinae, were checked frequently with photomicrographs.

TABLE I

Effect of Exposure of Trichina Larvae to Cobalt-60 or X-Ray on Persistence of Developing Trichinae in Small Intestine of Rats, and on Reproduction of Worms

Form of radiation	Dose of radiation	Average number of trichinae recovered from intestine at designated time after infection*		Average number of larvae recovered from skeletal muscles 35 days after infection
		3 days	6 days	
Cobalt-60	0	1,146 (3)†	2,222 (3)	193,000 (1)
	2,000 rep	1,613 (6)	2,213 (6)	144,000 (5)
	0	1,453 (3)	1,798 (3)	212,000 (2)
	4,000 rep	980 (6)	2,503 (6)	159,000 (3)
	0	2,270 (3)	2,847 (3)	267,000 (3)
	6,000 rep	3,366 (6)	2,596 (6)	11,050 (4)
	0	3,767 (3)	3,564 (3)	379,000 (3)
	8,000 rep	2,456 (6)	1,543 (6)	123 (3)
	0	3,609 (2)	1,228 (2)	455,000 (2)
	10,000 rep	1,700 (6)	435 (6)	20 (5)
	0	2,510 (3)	3,355 (3)	294,000 (5)
	12,000 rep	1,506 (5)	166 (5)	0 (6)
	0	1,534 (2)	1,775 (2)	300,000 (4)
	15,000 rep	17 (6)	11 (6)	0 (4)
	0	2,702 (2)	1,150 (2)	407,000 (4)
	18,000 rep	0–1 (6)	0–1 (6)	0 (4)
	0	2,647 (2)	1,836 (2)	
	30,000 rep	0 (6)	0 (6)	
X-ray	0	2,462 (2)	2,267 (2)	232,500 (4)
	1,000 r.	3,192 (6)	3,626 (6)	403,500 (4)
	0	2,890 (2)	3,025 (2)	257,000 (1)
	2,000 r.	2,513 (6)	3,463 (6)	19,500 (4)
	0	2,803 (2)	2,903 (2)	384,000 (4)
	4,000 r.	1,219 (6)	326 (6)	1 (4)
	0	2,375 (2)	1,900 (2)	471,000 (1)
	5,000 r.	408 (6)	1 (6)	0 (4)
	0	2,536 (2)	3,228 (2)	451,650 (3)
	6,000 r.	0 (6)	0 (6)	6 (3)
	0	1,117 (3)		361,250 (4)
	8,000 r.	0 (6)		0 (4)

* Small intestines of rats fed larvae irradiated with 1,000 to 4,000 rep cobalt-60 were washed with tap water; intestines of rats fed larvae exposed to 5,000 rep or more were washed with normal saline solution.

† Numbers within parentheses indicate number of rats tested. Each rat was fed 5,000 larvae.

Findings

Sexual Sterilization of Intestinal Trichinae

Diminished numbers of larvae or relatively few larvae were recovered from the skeletal muscles of test rats infected with larvae exposed to cobalt-60 in doses ranging from 5,000 to 10,000 rep and no larvae were recovered from the muscles of rats fed with larvae exposed to 12,000 rep or more cobalt-60. Similarly, a dose of 2,000 r. x-ray caused marked reduction in number of larvae recovered from the skeletal muscles, and a dose of 4,000 r. virtually prevented their development in the muscles (Table I and Text-figs. 1 and 2).

Table II

Time of Disappearance of Trichinae from Small Intestine of Rats Fed Larvae Exposed to Cobalt-60 or X-Ray

Type of radiation	Amount of radiation	Average number of trichinae recovered from intestine after designated time following infection			
		2 hr.	24 hr.	36 hr.	48 hr.
Cobalt-60	0 18,000 rep	1,363 (2)* 1,022 (2)	1,753 (2) 530 (2)		1,653 (2) 0 (3)
	0 18,000 rep		2,380 (2) 900 (3)	2,992 (2) 0 (3)	1,155 (2) 0 (2); 1 (1)
X-ray	0 6,000 r.	1,393 (4) 946 (4)	2,304 (4) 962 (4)		2,557 (3) 677 (3)
	0 6,000 r.	1,081 (2) 1,046 (2)	3,031 (2) 712 (2)	2,846 (2) 123 (3)	3,240 (2) 13 (3)
	0 7,000 r. 8,000 r.		2,132 (2) 963 (3) 881 (3)	2,292 (2) 4 (3) 4 (3)	2,100 (2) 0 (3) 0 (3)

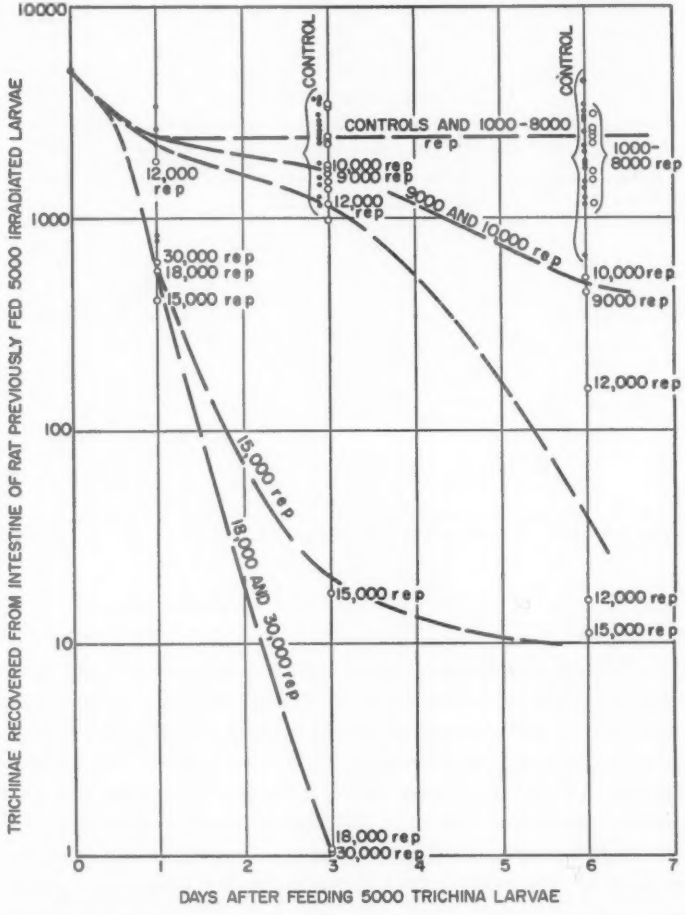
* Numbers within parentheses indicate number of rats tested. Each rat was fed 5,000 larvae.

Elimination of Trichinae from Intestine

The number of trichinae recovered from the small intestine 6 days after infection was diminished in rats fed larvae that had received doses of cobalt-60 of 8,000 rep or over and none were recovered from the intestine of rats fed larvae exposed to 18,000 rep or over. Comparable changes were noted after 4,000 r. and 5,000 r., respectively, of x-radiation (Table I). Three days following infection, the number of trichinae recovered from the intestine was reduced after 9,000 rep cobalt-60 and none were recovered after 18,000 rep; the number recovered after 4,000 r. x-ray was reduced and none were recovered after 6,000 r.

Table II lists data that point to elimination of most trichinae from

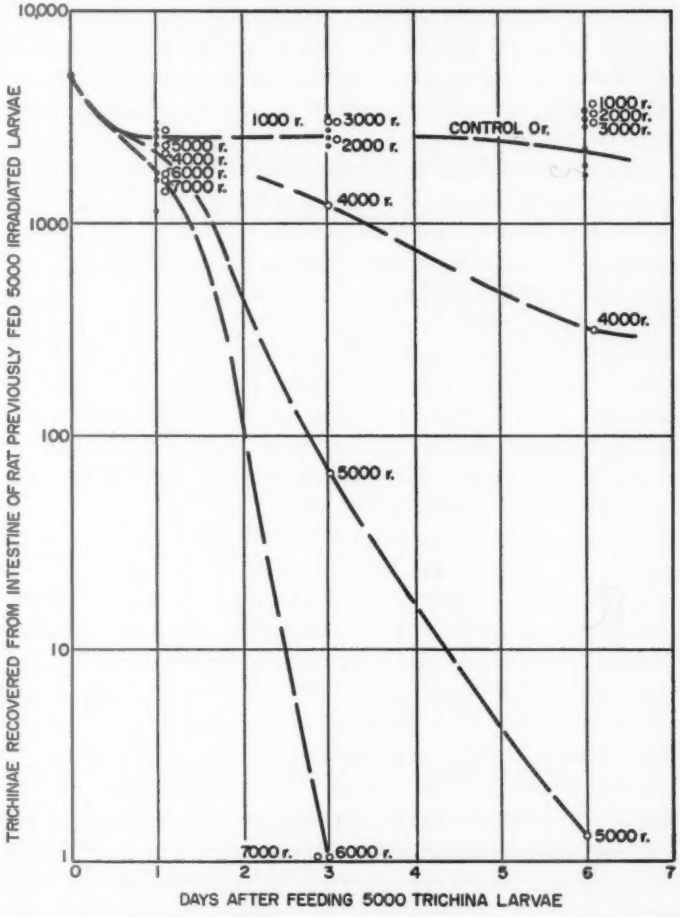
the intestine 24 hours after 18,000 rep cobalt-60 or after 7,000 r. x-ray, and to disappearance of all or nearly all trichinae 36 hours after feeding larvae exposed to these doses of radiation. Examination of microscopic sections of small intestine 2 to 48 hours after infection of rats fed larvae exposed to 18,000 rep cobalt-60 or 6,000 to 8,000 r. x-ray revealed that when trichinae were present, they were usually firmly imbedded in the mucosa, like trichinae developing from non-irradiated larvae. On direct examination, irradiated trichinae recovered from the small intestine were living.



Text-figure 1. Effect of exposure of trichina larvae to cobalt-60 on persistence of developing trichinae in small intestine of rats.

Morphologic Observations

Normal Structure. The fully developed adult female (Fig. 1) has a length of about 3 mm. and a width of about 65 μ. Its reproductive system (Fig. 2) consists of ovary, oviduct, seminal receptacle (distal portion of uterus), uterus proper, vagina, and vulva. The ovary is a single tubular structure located in the distal portion of the body. Developing germ cells arise from the ventral side of the ovary and pass proximally into the seminal receptacle through a constricted thick-walled oviduct. After copulation has taken place (as early as 30 hours



Text-figure 2. Effect of exposure of trichina larvae to x-ray on persistence of developing trichinae in small intestine of rats.

in the white rat),[9] the seminal receptacle of the female may be filled with sperms. Egg cells passing through the oviduct become fertilized after reaching the seminal receptacle. Egg cells in various stages of cleavage may be observed in the uterus within 12 to 24 hours after insemination. These eggs develop into slender worm-shaped embryos (Fig. 3), about 100 μ in length and 6 μ in diameter. The embryos move singly along a narrow muscular vagina and are expelled through the vulva as first-stage larvae. The larvae enter the lymphatics of the intestinal villi, are transported to the blood stream, and may be recovered from the blood of the host as early as 114 hours after infection.[9]

Adult male trichinellae (Figs. 1 and 4) may be distinguished from females by being shorter (about 1.5 mm.) and by the presence of two lateral, short, conical papillae (copulatory appendages) at the distal end of the body. The male reproductive system consists of testis, vas deferens, seminal vesicle, and ejaculatory duct (Fig. 5). The testis begins as a blunt structure at the distal end of the body, extends proximally as far as the base of the esophagus, then turns distally as the vas deferens which becomes expanded as the seminal vesicle and this in turn leads to the ejaculatory duct. The duct joins the intestine to form a cloaca which opens exteriorly at the distal end.

Insemination of adult females usually begins 32 to 36 hours after the host is fed excysted larvae.[9] At 36 hours, 14 of 15 females examined were inseminated, and at 48 hours all females examined were inseminated. At 72 hours sperms filled the seminal receptacle, occupying as much as one eighth of the volume of the uterus; sperms also were distributed throughout the remainder of the uterus. The egg cells in the uterus were spherical, tightly packed, and were undergoing cleavage. At 120 hours, all of 100 adult females examined were inseminated. In general, females contained increasing quantities of sperms in the seminal receptacle from 32 hours to 144 hours, suggesting that more than one insemination had taken place. Males had large numbers of sperms in the vas deferens and seminal vesicle.

Examination of groups of 15 female intestinal trichinae, recovered from rats at various intervals from 36 to 120 hours after infection, revealed cleaving eggs or worm-shaped embryos within the uterus in the numbers shown in Table III, in which it appears that the number of egg cells undergoing cleavage is maximal at 4 days. At 5 days a considerable percentage of these have developed into worm-shaped embryos and some of the latter have already entered the circulation of the host.[9]

Morphologic Changes. Measurement of intestinal trichinae revealed appreciable reduction in body length of irradiated male and female trichinae, as compared with non-irradiated trichinae. Prior to 24 hours the length of the intestinal trichinae could not be measured accurately,

TABLE III

The Numbers of Cleaving Eggs and of Worm-Shaped Embryos in Group of Fifteen Female Trichinae Recovered at the Stated Intervals after Infection

Hours after infection	Number of eggs in cleavage		Number of worm-shaped embryos	
	Range	Average	Range	Average
36	27–63	44		
48	101–152	122		
72	100–191	143		
96	132–321	209	24–51	35
120	121–198	144	52–102	75

owing to the partial coiling of living worms or to the C-shape or S-shape of fixed trichinae.

After exposure of larvae to 6,000 r. x-ray, growth of intestinal trichinae was inhibited at 24, 36, and 48 hours after infection. This was indicated by the average body length of formalin-fixed trichinae at the designated intervals after infection (Table IV).

TABLE IV

Body Length of Intestinal Trichinae Recovered at Stated Intervals after Exposure of Larvae to 6,000 r. X-Ray, Compared with Non-Irradiated Controls

Trichinae	Hours after infection	Length of females	Length of males
		μ	μ
Non-irradiated	24	1,407 (10)*	1,214 (10)
Irradiated	24	1,073 (10)	994 (10)
Non-irradiated	36	1,521 (10)	1,259 (10)
Irradiated	36	910 (10)	750 (1)
Non-irradiated	48	1,859 (10)	1,260 (10)
Irradiated	48	1,177 (10)	981 (8)

* Numbers in parentheses indicate the number of trichinae measured.

Reduction was noted 3 days after exposure of larvae to at least 5,000 rep cobalt-60 or 2,000 r. x-ray and 6 days after exposure of larvae to at least 8,000 rep cobalt-60 or 2,000 r. x-ray. No trichinae were recoverable from the intestine 3 days after exposure to 18,000 rep cobalt-60

or 6,000 r. x-ray, and 6 days after exposure to 18,000 rep cobalt-60 or 5,000 r. x-ray (Table V).

At 96 hours after infection with larvae exposed to 4,000 r. x-ray, the average length of 15 formalin-fixed, irradiated females was 1.5 mm. compared to 2.4 mm. for 15 non-irradiated females.

TABLE V

Effect of Cobalt-60 or X-Ray on Length of Body of Adult Trichinellae 3 and 6 Days after Irradiation of Larvae (Each Figure Represents Average Length, in Microns, of 10 Adult Worms Fixed in Hot Formalin)

Type of radiation	Amount of radiation	Female trichinellae		Male trichinellae	
		3 days	6 days	3 days	6 days
Cobalt-60	0	1,812	1,977	1,184	1,275
	3,000 r.	1,856	1,982	1,238	1,171
	0	2,076	1,890	1,379	1,286
	6,000 r.	1,626	1,761	1,214	1,208
	0	1,887	2,077	1,259	1,313
	9,000 r.	1,377	1,551	1,070	1,165
	0	1,911	1,875	1,260	1,200
	12,000 r.	1,292	1,341	980	1,350*
	0	1,751	1,916	1,172	1,223
	15,000 r.	1,321	1,501	1,070	1,185
	0	1,974	2,001	1,283	1,287
	18,000 r.	—	—	—	—
X-ray	0	1,929	1,804	1,179	1,255
	1,000 r.	1,766	1,911	1,047	1,226
	0	2,039	2,403	1,308	1,380
	2,000 r.	1,658	1,732	930	1,226
	0	2,014	1,996	1,346	1,304
	4,000 r.	1,278	1,388	937	1,130
	0	2,063	1,962	1,411	1,301
	5,000 r.	1,238	—	1,300	—
	0	1,846		1,204	
	6,000 r.	—		—	

— Indicates that no trichinae were recovered from the intestine.

* Only 1 male worm was recovered; the body was swollen and the testis was irregular in shape.

Table VI summarizes the morphologic changes observed in living, non-irradiated, intestinal trichinae and in those at various intervals from 2 to 144 hours after irradiation with 2,000 to 6,000 r. x-ray (Figs. 1 to 12). In every instance larger doses of cobalt-60 (approximately three times those of x-ray) were required to produce the same effects as were produced by x-radiation (Figs. 13 to 17).

Partial sexual sterilization of trichinae is indicated by reduction in

number of worm-shaped embryos in the uterus of female worms (some of the female worms had no embryos) recovered from the intestines of rats 6 days after infection with irradiated larvae; and by reduction in number of larvae of the second generation recovered from skeletal muscles of rats 35 days after infection (Fig. 6; results in Table I after 6,000 rep cobalt-60 or 2,000 r. x-ray). Complete sterilization is indicated by absence of worm-shaped embryos or sperms in the uterus of all female intestinal trichinae 6 days after infection, and by absence of larvae in the skeletal muscles of rats 35 days after infection.

Ordinarily, non-irradiated trichinae are largely or completely eliminated from the small intestine of white rats 16 days after infection. Direct observations and the findings listed in Table I indicate that appreciable numbers of living but sexually sterile trichinae remain in the intestine for 3 to 6 or more days after infection. Even after 30,000 rep cobalt-60 or 8,000 r. x-ray a considerable number of trichinae may be recovered 24 hours after infection. Alicata[7] recovered trichinae from the intestine of rats 24 hours after infection with larvae exposed to x-ray in doses up to 600,000 r.; but was not able to recover any 24 hours after exposure to 700,000 r., a dose that he regarded as lethal.

Despite the production of sexual sterilization by radiation, some trichinae remain alive in the intestine for 1 to 6 or more days. With increasing doses of radiation the worms are lost in increasing numbers and at shorter intervals after radiation. Earlier and greater elimination from the intestine is correlated with greater damage produced by larger doses of radiation.

No morphologic change was noted either in males or in females 12 to 18 hours after 4,000 r. x-radiation. It therefore seems that a latent period following infection is required before the morphologic changes become apparent in irradiated trichinae. Twenty-four hours after infection with larvae exposed to these doses of x-radiation, reduction in size and other changes were obvious, and yet most of the trichinae fed were recoverable from the intestine at this time.

Some measurements of the length of the body of fixed intestinal trichinae, 3 and 6 days after larvae had been exposed to various doses of cobalt-60 or of x-ray, are given in Table V. Measurements of the width of the body varied only slightly from those of control worms; measurements of the length and width of the gonads are not included inasmuch as the shrinkage of the gonads caused by irradiation was not uniform and hence these measurements did not appear to represent a true index of the damage caused.

Maximum decreases in body length at 3 days were found after

TABLE VI

Morphologic Changes Observed in Living Trichinellae Recovered from Small Intestine of Rats Fed Larvae Exposed to X-Irradiation

Hours after infection	Dose of x-ray applied to larvae fed to rats				
	0 (controls)	2,000 r.	3,000 r.	4,000 r.	6,000 r.
2					No changes.
12				No changes.	
18	Molting.			No changes. Molting.	
24		No changes.		Little differentiation of uterus and vulva. Membrane of ovary and uterus partially collapsed. Egg cells in ovary degenerating, granular.	Reduction of length; gonads and stichosomes damaged. Ovary partially damaged; development of uterus retarded; intestine filled with fluid.
36	14 of 15 females inseminated, egg cells unfertilized. Males: seminal vesicle and vas deferens full of sperms.		Females: 3 of 15 inseminated, egg cells in cleavage (2-8 cell stages.) Distal portion of ovary damaged. Non-inseminated females: molting, little increase in length of body, slight differentiation of uterus; no cleavage of egg cells. In some, vulva obstructed. Males: molting; in some, cells detached from testis, undergoing liquefaction; sperms in seminal vesicle and vas deferens.	Degenerating egg cells in ovary. In one male, seminal vesicle and vas deferens filled with sperms.	Growth stunted. Most of ovary destroyed, contained liquid material with cellular fragments; egg cells fragmented. Stichosome damaged, granular. Males: testis damaged.
48	All females inseminated; egg cells in uterus in cleavage.	17 of 112 females inseminated. Males appeared normal.		None of 15 females inseminated. Seminal receptacle filled with débris of degenerating egg cells.	Worms stunted. Most of ovary destroyed. Males: testis degenerating, of fluid appearance, cells fragmented.
72	All females inseminated, uterus full of spherical egg cells in cleavage. Males had numerous sperms.	Some females inseminated. Egg cells in cleavage, some degenerating. Males appeared damaged.	4 of 100 females inseminated but had few sperms, poorly developed egg cells. Non-inseminated females: distal portion of ovary damaged, uterus under-developed, some egg cells malformed. In some, stichosome shrunken. In few, vagina and vulva obstructed.	Length reduced. Degeneration of gonads, more severe in females. Thickening of cuticle, occlusion of vulva.	Only occasional female recovered.
96		7 of 25 females inseminated. In few, ovary damaged, cellular débris in uterus. Cleavage at 48-72 hr. stage; some eggs disintegrating at 32- or 64-cell stage.	Distal portion of ovary damaged. Egg cells unfertilized and degenerating, especially in seminal receptacle; about one third of uterus occupied by cellular débris. Vulva completely obstructed.	Growth stunted. Cuticle irregularly swollen. Ovary shrunken, malformed; had few egg cells. Vulva often occluded by granular débris.	None recovered.

120	All of 100 females inseminated; worm-shaped embryos in 15.	Few worm-shaped embryos (3–20), of normal appearance. In some females, ovary collapsed; decreased number of egg cells, some malformed.	Growth stunted. Of 100 females, only 1 inseminated; eggs in 2–16 cell stages. Distal portion of ovary, granular degeneration. In some, obstruction of vulva and vagina; cuticle nodular. Males: testis of few, hydropic and granular degeneration; some stichocytes malformed, vacuolated.	Females: growth stunted, ovary shrunken.	None recovered.
144		Stichocytes shrunken, some vacuolated. Females: uterus small, wall thickened; egg cells unfertilized or degenerating; few worm-shaped embryos. Vulva abnormal or sealed. Distal portion of intestine thickened. Males: few or no sperms; testis, vas deferens, intestine, granular.	Females sterile.	Ovary fused with uterus; both filled with granular material. Cuticle in distal half of body thickened up to 10.5 μ (about twice normal), often nodular. Vulva often occluded.	None recovered.

exposure to 8,000 rep cobalt-60 and at 6 days after 10,000 rep. The average length of males irradiated with x-rays was also decreased for each dose of x-radiation given. However, it must be noted that non-irradiated adult female trichinae are naturally larger than males and at 6 days owe part of their greater size to the presence in the uterus of a large amount of sperm and of many developing young embryos. Hence, a greater reduction in body length of females compared to that of males was to be expected after irradiation, both at 3 and 6 days.

In seeking a possible explanation for the difference in amounts of radiation of cobalt-60 and of x-ray that are necessary to produce a given effect, it may be noted that sexual sterilization was produced by 12,000 rep cobalt-60 applied at a radiation rate of 1,333 rep per minute (approximately 9 minutes) and by 4,000 r. x-ray applied at a radiation rate of 312 r. per minute (approximately 12.8 minutes). One may wonder if the difference in the dose of the two forms of radiation may be explained by a constant factor connected with the radiation rates. In previous experiments, we were able to produce sexual sterilization of intestinal trichinae with approximately 4,000 r. x-ray applied at the rate of 1,000 r. per minute; with approximately 12,000 rep of mixed fission products (believed to be predominantly cesium-137) applied at rates ranging from 60 to 20 rep per minute;[10] and with approximately 14,000 rep cesium-137

applied at a rate of 11.5 rep or less per minute.[11] Consequently, the total doses required to produce sterilization do not appear to be strongly influenced by the radiation dose-rate. We plan to investigate the origin of the difference in biologic effectiveness of the 250 kv. and cobalt gamma rays as determined by the difference in doses required to produce sexual sterilization of trichinae. It should be mentioned, however, that we found no difference in the dose of radiation necessary to kill trichina larvae *in vitro* (approximately 750,000 rep for cobalt-60[2] and 750,000 r. for 250 kv. x-ray[1]).

Summary

Excysted larvae of *Trichinella spiralis* were exposed to various doses of cobalt-60 and of x-ray and then fed to white rats to determine the effect of these forms of radiation on prevention of reproduction of the worms, on the rate of elimination of the worms from the intestine of the host, and on the length of the body of intestinal trichinae at various intervals after infection. In addition, direct observations were made, on living and fixed trichinae, of morphologic changes appearing at various intervals following infection with larvae exposed to different doses of radiation.

In each instance in which the effects of cobalt-60 and x-ray were compared, it was found that the dose of cobalt-60 necessary to produce a given effect was approximately three times the dose of x-ray. The striking difference in dosage of the two forms of gamma radiation is believed to be caused by the difference in distribution of ionization. This is now being investigated.

Exposure of trichinella larvae to 5,000 to 10,000 rep cobalt-60 or to 2,000 or 3,000 r. x-ray greatly reduced their reproductivity; doses of 12,000 rep and over of cobalt-60 and 4,000 r. and over of x-ray produced sexual sterilization of practically all trichinae. In rats fed larvae exposed to 18,000 rep cobalt-60 or 7,000 r. x-ray, all or nearly all trichinae were eliminated from the small intestine within 36 hours after infection.

During the first day (approximately 18 hours) following radiation there is a latent period during which few or no morphologic changes are apparent.

Morphologic changes were noted in female trichinellae at various intervals from 24 to 144 hours after infection of white rats with larvae exposed to cobalt-60 or to x-ray. Irradiation of trichina larvae (with 6,000 rep or more of cobalt-60 or 2,000 r. or more of x-ray) produced decreases in body length, when measured 3 and 6 days after irradiation.

Exposure to 18,000 rep cobalt-60 or 6,000 r. x-ray resulted in severe stunting of growth as early as 24 hours after infection. Six days after exposure to 2,000 r. x-ray, the gonads of both males and females showed evidence of degeneration and only a small proportion of females were inseminated. After 3,000 r. the damage was greater and only an occasional female was inseminated (with only few sperms). The vulvas of some females were obstructed. After 4,000 r., practically no females were inseminated. Growth of males and females was severely stunted. The females exhibited extensive degeneration and shrinkage of the ovaries, and thickening and nodular swelling of the cuticle. It seems likely that granular degeneration and disintegration of the egg cells was a factor in causing obstruction of the vagina and vulva.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

All figures are of intestinal stages of *Trichinella spiralis*. Figures 1 and 12 to 17 inclusive are of unstained living worms; Figures 2 to 11 inclusive are of worms fixed in 70 per cent alcohol and glycerine, stained with alum cochineal, and counterstained with fast green.

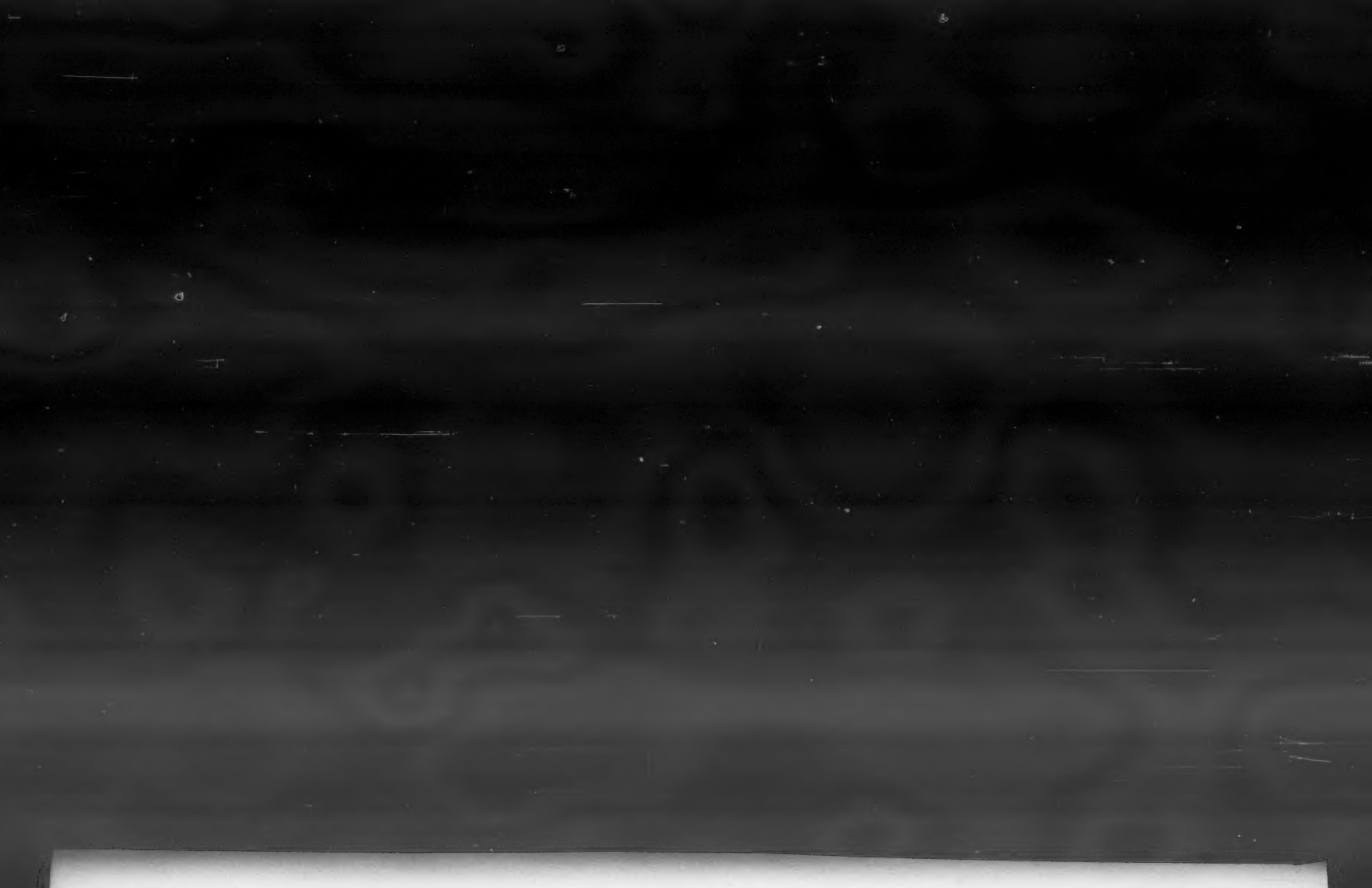
FIG. 1. Living adult male *Trichinella spiralis* (upper) and female (lower) recovered from small intestine of white rats 4 days after infection. × 85.

FIG. 2. Distal half of non-irradiated female, 6 days after infection, showing ovary (ov), seminal receptacle (s.r.) representing distal portion of uterus, and fertilized egg cells in uterus (ut). × 210.

FIG. 3. Midportion of non-irradiated female, 6 days after infection, showing worm-shaped embryos within uterus. × 260.

FIG. 4. Distal half of non-irradiated male, 6 days after infection. × 220.

FIG. 5. Distal half of male, 6 days after irradiation with 2,000 r. x-ray. t indicates testis; v.d., vas deferens; s.v., seminal vesicle; e.d., ejaculatory duct. Sperms may be noted within testis and vas deferens. × 230.



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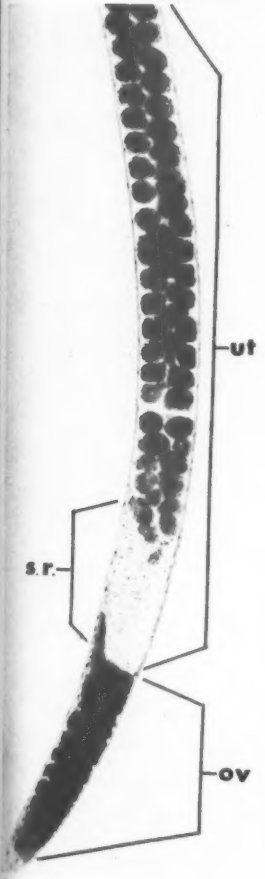

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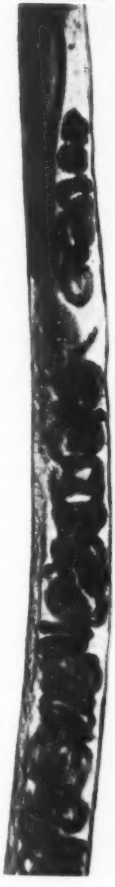

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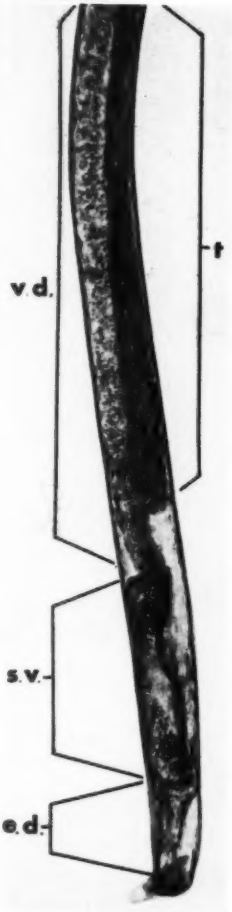

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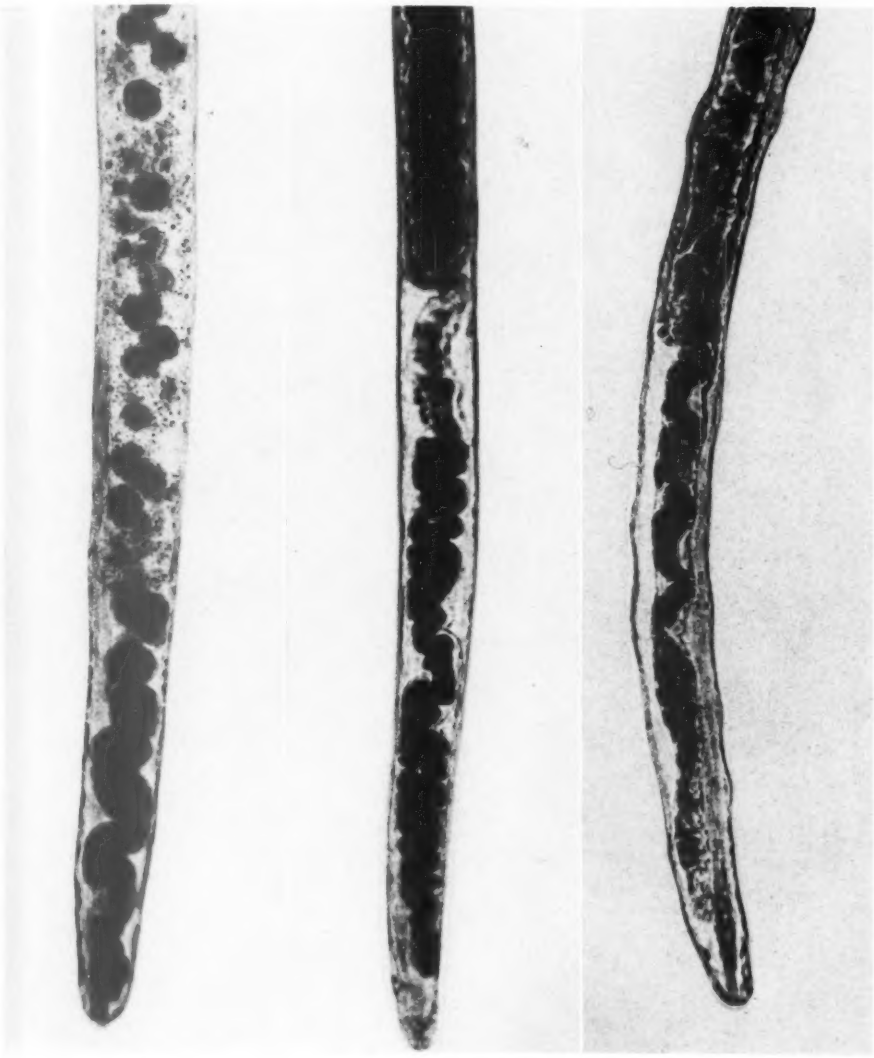
FIG. 6. Midportion of female trichina 6 days after exposure to 2,000 r. x-ray. The number of egg cells in cleavage is reduced and only a few worm-shaped embryos are present in the uterus. × 310.

FIG. 7. Distal half of female trichina, 6 days after infection with larvae exposed to 2,000 r. x-ray. The uterus is filled mainly with unfertilized egg cells. One egg cell (large rounded cell at left of center) is undergoing cleavage. No worm-shaped embryos are seen. × 260.

FIG. 8. Distal half of female 6 days after irradiation with 2,000 r. x-ray. The ovary has undergone shrinkage, there is much granular material in the distal portion of the uterus, and fertilized egg cells may be seen in the uterus. × 310.



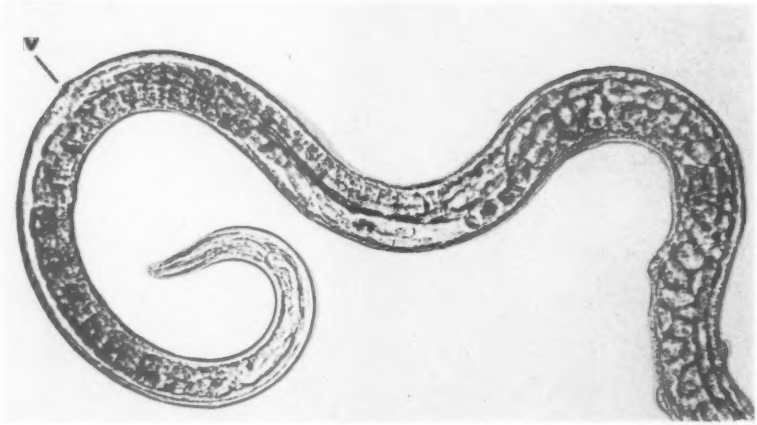
9 10 11

FIG. 9. Distal third of female trichinella, 6 days after infection of rat with larvae exposed to 2,000 r. x-ray. The ovary is irregular. The uterus contains a reduced number of egg cells in cleavage, some of which appear to be disintegrating. No definite worm-shaped embryos are seen. × 310.

FIG. 10. Distal third of female trichinella, 6 days after infection of rat with larvae irradiated with 2,000 r. x-ray. Upper third of figure is occupied by distal portion of uterus. The wall of the uterus is thickened and the lumen contains immature egg cells but no sperms. × 310.

FIG. 11. Distal third of female trichina, 6 days after infection, developing from larva exposed to 4,000 r. x-ray. The cuticle shows nodular thickening (not an artifact of fixation). The ovary (in the lower 5/8ths of the figure) is deformed and contains a reduced number of egg cells which are clumped and stain intensely. The upper 3/8ths of the field is occupied by the distal portion of the uterus; its wall is thickened and its lumen contains immature egg cells but no sperms. × 310.

12



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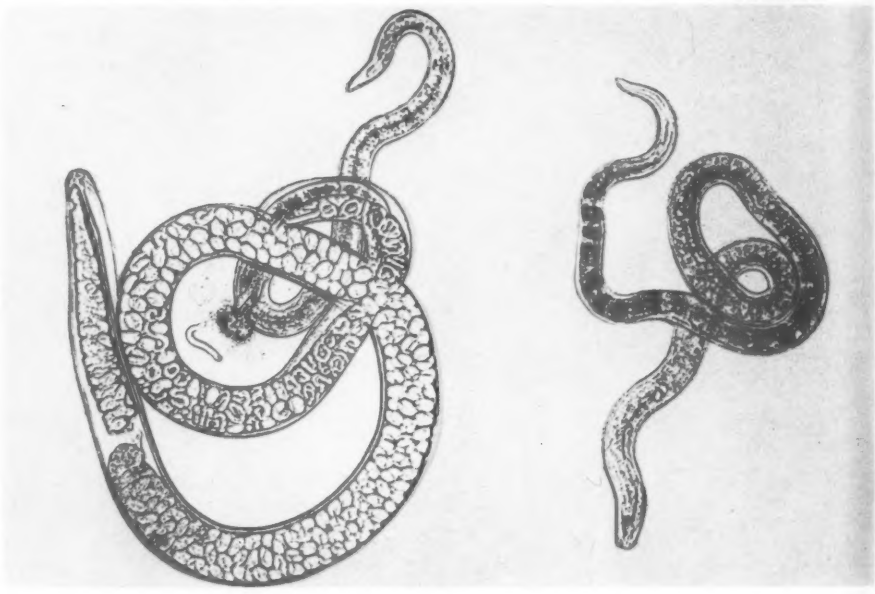

FIG. 12. Proximal two thirds of living female trichina, 6 days after 4,000 r. x-ray. The vulva (v) is occluded, and nodular thickening of cuticle is seen in the right portion of the field. × 280.

FIG. 13. At the left is a non-irradiated gravid female trichina that was recovered from the intestine of a rat 6 days after infection; a larva has just been extruded from vulval opening. × 145. At right is a sexually sterile adult female trichina of the same age. × 145. This trichina was exposed in its larval excysted state to 10,240 rep cobalt-60. There is stunting of growth and extensive degeneration of cell body, uterus, and ovary. (Reproduced by permission from: *J. A. M. A.*, 1954, **154**, 653–658.)

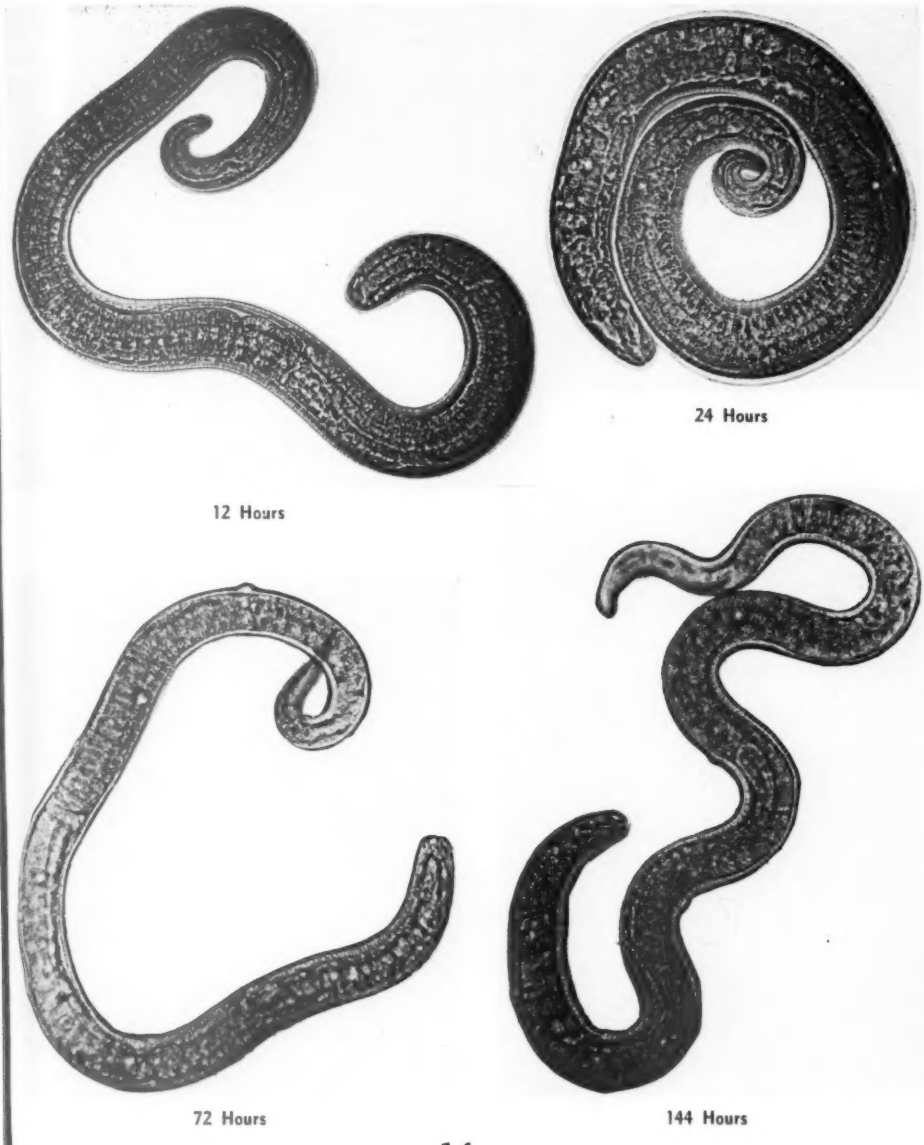

FIG. 14. Living female intestinal trichinae at various intervals from 12 hours to 144 hours after exposure of larvae to 4,000 r. x-ray. × 240.

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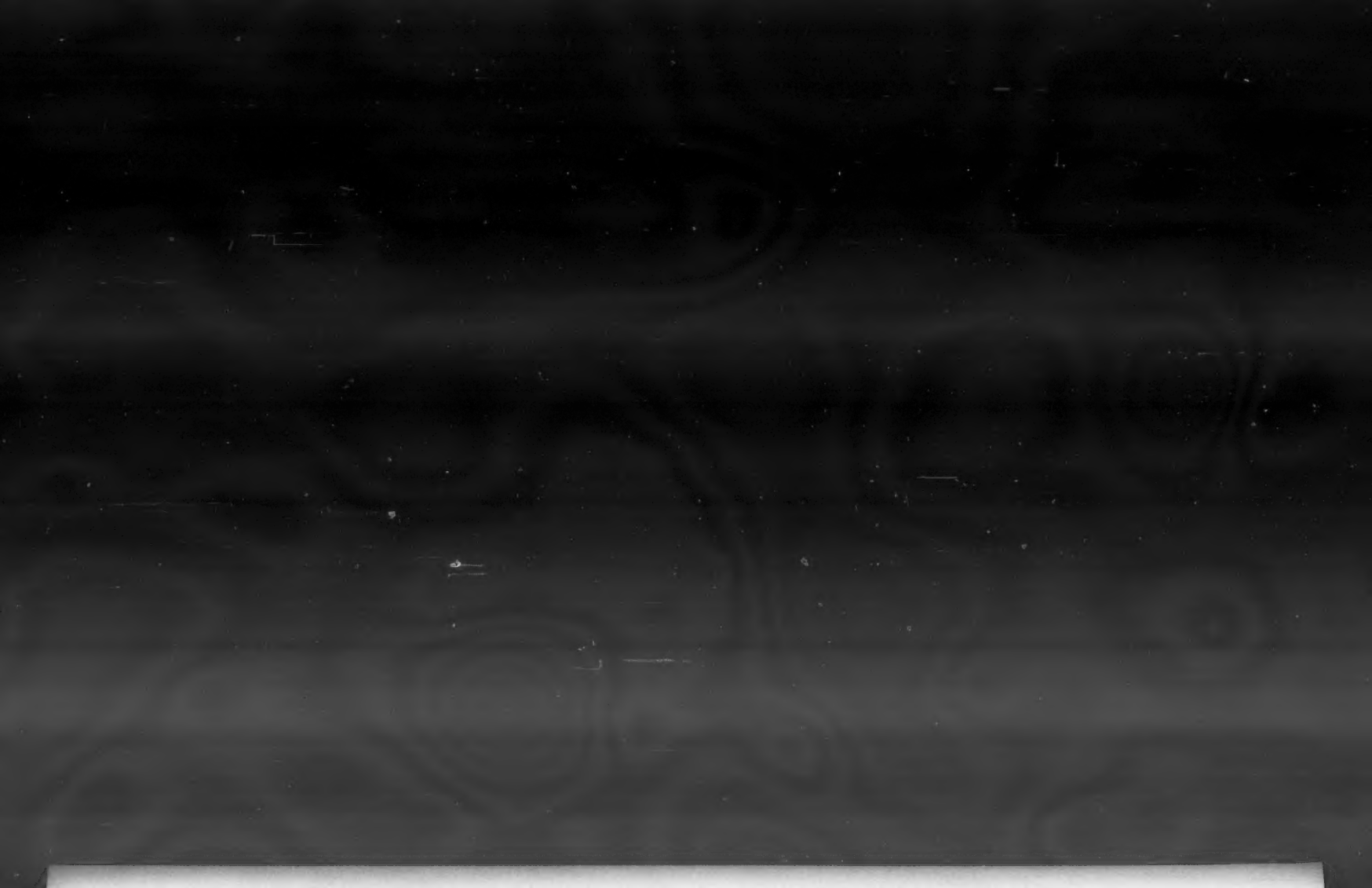
FIG. 15. Living female intestinal trichinae 72 hours after exposure of larvae to various doses (2,000 r. to 6,000 r.) x-ray. × 195.



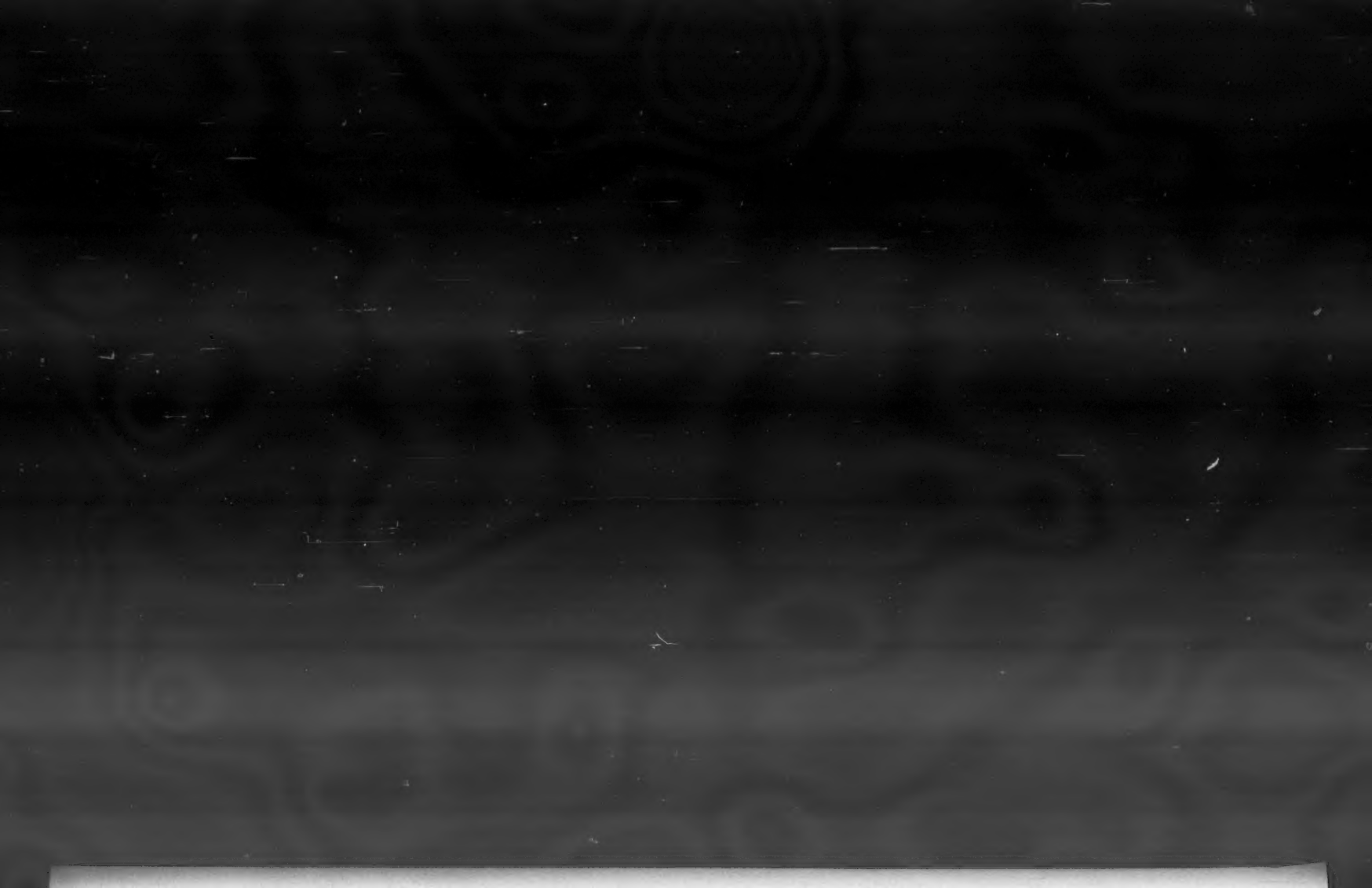
No X-ray

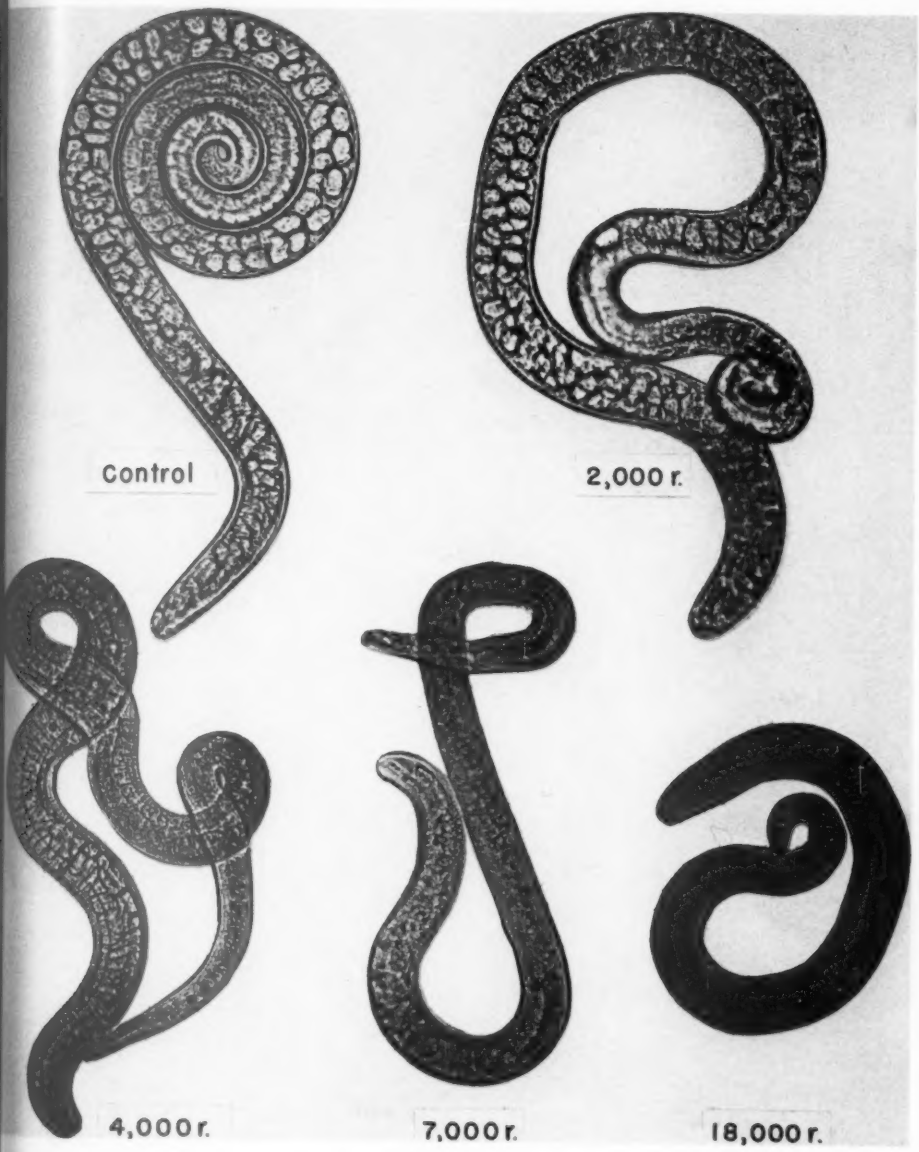
2,000 r.

4,000 r.

6,000 r.

FIG. 16. Living female trichinae 72 hours after exposure of larvae to radiation of cobalt-60 (2,000 rep to 18,000 rep). × 210.

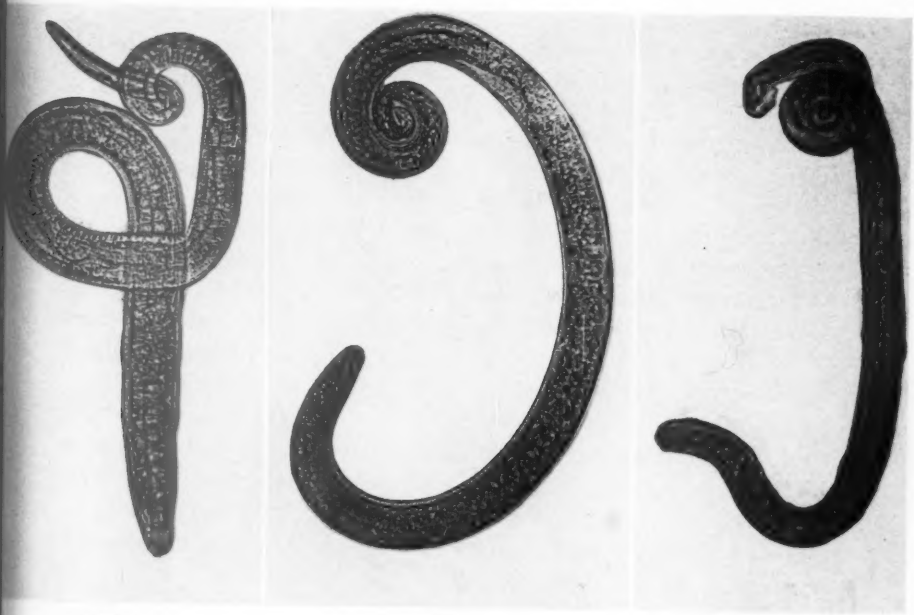




16

FIG. 17. Living female intestinal trichinae 144 hours after exposure of larvae to radiation of cobalt-60 (4,000 rep to 15,000 rep). × 170.

Control 4,000 r.



7,000 r. 12,000 r. 15,000 r.

TUBERCULOSIS INDUCED BY DROPLET NUCLEI INFECTION

Its Developmental Pattern in Hamsters in Relation to Levels of Dietary Protein *

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The development by man of effective resistance to progressive infection by tubercle bacilli is believed to be influenced to a considerable extent by levels of dietary protein and of vitamins A and C.[1-4] This opinion is based largely upon clinical and epidemiologic studies, although to some extent it finds support in observations on tuberculosis among birds at the Philadelphia Zoological Garden[5] and in experimental studies of diet and tuberculosis in mice.[6,7] However, experiments on diet and tuberculosis in rats have given conflicting results.[8,9]

An opportunity for more definitive studies of tuberculosis and nutrition has been provided by Wells's development of apparatus and techniques for quantitative study of droplet nuclei infection.[10-12] This apparatus and these techniques allow precise control of the number of tubercle bacilli that will be inhaled and deposited in the lungs. Thus, the initial intensity of infections may be predetermined at any desired level and held at that level through any number of experimental groups.[11,13]

Tuberculosis induced by droplet infection in rats, mice, guinea-pigs, hamsters, and rabbits follows a highly uniform developmental pattern in all host-parasite combinations for about 3 weeks. Thereafter, the rate and pattern of development of tubercles in the lungs and the rate of progress of the disease as a whole differ with the host-parasite combination. Within a given host-parasite combination, however, the reaction to infection is still highly uniform for a time before individual variations become evident.[14,15]

If dietary protein be a factor in the rate at which resistance develops or in its level of effectiveness,[16] its influence should be more easily detected in host-parasite combinations that are characterized by prolonged periods of uniform reaction. In experimental studies, these occur to the best advantage in guinea-pigs and hamsters that have been subjected to inhalation infection by small numbers of virulent bacilli

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of human origin, and in rats subjected to inhalation infection by numbers of virulent bacilli of bovine origin.[15]

The present report describes the effects of three levels of dietary protein upon the progress of tuberculosis induced in Syrian hamsters by droplet nuclei infection. Simultaneous experiments with rats and guinea-pigs will be presented later. These reports will amplify the summaries of certain experiments that have been published earlier.[17]

Materials and Methods

Syrian hamsters were supplied by a dealer who maintains a highly vigorous breeding stock. They were delivered to the laboratory at about 4 weeks of age with each litter as a unit accompanied by its pedigree for three generations. Identities were maintained by separate housing in small, numbered cages. The colony diet was fed until all litters attained mean average weights of at least 100 gm., at about 8 weeks of age. Weights were recorded at biweekly intervals throughout the study. Each diet group contained approximately 50 animals, the exact number depending upon a convenient combination of litters. All groups were allowed at least 6 weeks for adjusting to the diets before infections were induced, and were continued on the diets until killed for study.[18]

Infections were induced by means of Wells's apparatus and techniques.[10,11,13] As insurance against possible changes in virulence of the test organism, not less than two diet groups and usually three were infected from each suspension of tubercle bacilli during a period of no more than 5 hours. Aqueous suspension has not altered virulence and viability of the organisms during this interval.[13]

As a further check on the virulence of the organism and also on the intensity of the initial infection (i.e., the number of initial tubercles), 10 to 12 hamsters of each diet group were killed 75 to 80 days after infection. The mean number of initial tubercles in these samples of all diet groups was found to be less than 10, and in 15 of the 18 groups, less than 5. These values corresponded closely to the number of bacilli calculated to have been inhaled, again demonstrating that a high percentage of all bacilli inhaled were deposited in the lungs and induced tubercles.

Cultures of the H37Rv strain were supplied by the Standard Culture Depot of the National Tuberculosis Foundation, Trudeau, New York, and maintained on solid and liquid media.[11,12] The aqueous suspensions of bacilli used to induce infection were prepared from liquid cultures after 6 to 18 days of incubation. The rate of tubercle development was not related to the age of the culture.

The first culture was received on December 3, 1948. During November, 1952, its characteristics of growth changed abruptly. Since this change could have been associated with a loss of virulence, the culture was replaced by a second one on December 26, 1952. The virulence of this corresponded completely with that of the first, as judged by the size of the initial tubercles 75 to 80 days after infection, until February, 1954, when its virulence also changed abruptly. However, reduced virulence was not associated with visible changes in its growth characteristics. Meanwhile, it had been used to induce infection in three groups of hamsters before loss of virulence was recognized. A third culture was used for the terminal experiments of this series.

After testing several other mixtures, the isocaloric diets shown in Table I were devised and found to be about equally acceptable to hamsters, guinea-pigs, and rats, and to induce similar responses in these animals.

TABLE I
Test Diets *

	A	B	C
Salt mixture no. 12[19]	4.0%	4.0%	4.0%
Vitamin mixture†	4.0	4.0	4.0
Cod liver oil (USP)	2.0	2.0	2.0
Wheat germ oil	1.0	1.0	1.0
Cottonseed oil	4.0	4.0	4.0
Choline-cerelose (1:9)	2.0	2.0	2.0
Pulverized beet pulp	12.0	12.0	12.0
Pulverized rolled oats	25.0	15.0	5.0
Cystine-casein (1:50)	25.0	15.0	5.0
Cerelose‡	10.0	34.0	47.0
Corn syrup	11.0	7.0	14.0

* We gratefully acknowledge the interest and advice of Dr. J. H. Jones, Professor of Biochemistry, School of Veterinary Medicine, University of Pennsylvania.

† 2 gm. each of thiamine, riboflavin, and pyridoxine; 12 gm. each of calcium, pantothenate, inositol, and nicotinic acid; 1.2 gm. of folic acid; 1 gm. of menadione; 0.08 gm. each of biotin and vitamin B_{12}; cerelose to 4,000 gm.

‡ Cerelose, trade name for dextrose prepared from corn.

Corn syrup was used as part of the carbohydrate to increase the cohesiveness and reduce wastage. Estimated levels of protein were approximately: diet A, 30 per cent; diet B, 17 per cent; and diet C, 6 per cent. Diets were mixed at intervals of 7 to 10 days and stored in inert metal containers at about 0° C. Food supplies in the cages were renewed at 2-day intervals.

These mixtures were satisfactory for hamsters and rats and, when supplemented by adequate amounts of ascorbic acid, served equally well for guinea-pigs. Diets A and B allowed reproduction and supported growth and maturation of young. The average body weight of animals on diet C usually became stable at about 85 to 90 per cent of that of animals on diet A. Animals on diet B were somewhat heavier than those on diet A. However, a more detailed comparison of body weights hardly seems justified since progressive tuberculosis often was associated with rapid loss of weight in animals on diets B and C.

The animals were killed for study 75 to 80, 125 to 130, 150 to 155, 175 to 180, and 200 days after infection. They were chloroformed and

exsanguinated by cutting axillary vessels, after which the extent and characteristics of tuberculosis in the lungs, thoracic lymph nodes, and spleen were recorded. The thoracic viscera were next removed en bloc. The lungs were allowed to collapse by gravity and were then filled with Bouin's fluid through the trachea and immersed in this fixative, with other tissues that were taken for microscopic study. Tissues were embedded in paraffin and stained to demonstrate the inflammatory reaction, the bacilli, and the connective tissue. All sections were cut and stained by one technician.

Results

Tuberculosis induced by bacilli of high virulence (figs. 1 to 14)

Tuberculosis developed at a highly uniform rate for about 130 days in hamsters of all diet groups that had inhaled the more highly virulent bacilli. Thereafter its rate of progress was much more variable, but always most rapid in animals on diet C (6 per cent protein).

Variations in the rate of progress of the disease after 130 days were not related to sex. In fact, these variations were quite as great among litter mates of either sex as among less closely related animals on the same diet. The range of variation seemed to be greatest among animals on diet B (17 per cent protein). Indeed, tuberculosis in this diet group almost bridged the difference between the patterns of disease in animals on diet C (6 per cent protein) and diet A (30 per cent protein). But, since this study has been concerned with average patterns of tuberculosis and their relations to levels of dietary protein, attention will be given mainly to the more significant lesions of animals on diets C and A.

Macroscopic Appearances

From 75 to 130 Days after Infection (Figs. 1 and 2). Initial tubercles of all animals killed 75 to 80 days after infection were sharply defined nodules about 5 mm. in diameter. At this time, too, secondary tubercles of the thoracic lymph nodes also measured about 5 mm. in diameter in all animals. Secondary tubercles of the lungs and spleen were much smaller and were irregularly distributed among the animals. In fact, the lungs of the animals on diet A contained few secondary tubercles, whereas these lesions were numerous in the spleen. This distribution was reversed in the lungs and spleen of animals on diet C (Figs. 1 and 2).

The second sample from these diet groups (125 to 130 days after infection) found little change in the size of the initial tubercles or in

the size and distribution of the secondary lesions. This relatively quiescent stage of the disease was followed shortly by one in which progress seemed to have accelerated rapidly in animals on diet C and in many of the animals on diet B.

From 130 to 155 Days after Infection (Fig. 3). Initial tubercles of animals on diet C measured upwards of 10 mm. in diameter at 150 days. Secondary tubercles of the thoracic lymph nodes and spleen also had enlarged considerably within this interval of about 20 days. Often, too, the lungs contained numbers of minute (miliary) tubercles, as well as scattered secondary lesions that were believed to have originated much earlier.

From 155 to 200 Days after Infection (Figs. 4, 5, and 6). From 150 to 180 days after infection one or more initial tubercles in the lungs of all animals on diet C, and of many on diet B, apparently continued to expand irregularly. Within this interval initial tubercles also underwent more or less central liquefaction, with endobronchial tuberculosis becoming increasingly pronounced by 180 days. Often, too, the more advanced stages of endobronchial disease were accompanied by severe miliary tuberculosis of the lungs, liver, and spleen, and by further increase in size of the larger secondary tubercles in the lymph nodes and spleen. The more rapidly progressive forms of tuberculosis in animals on diet C and B were regularly associated with precipitous losses in body weight, and with pronounced distortion of the lobular structure of the liver by tubercles.

In occasional animals on diet C the spleen was even smaller than at 75 days, and tubercles of the thoracic lymph nodes were no larger than at this earlier stage. Still, even in these animals, the initial tubercles had undergone central liquefaction with endobronchial spread of the infection, which corresponded to the behavior of tuberculosis in vaccinated rabbits.[20]

In contrast to the pattern of progressive tuberculosis of animals on diet C (6 per cent protein), the process seen 75 days after infection in animals on diet A (30 per cent protein) seemed to have remained unchanged or, possibly, to have undergone more or less regression during the following 100 days. Thus, in about half the animals on diet A killed 180 days after infection, all lesions were smaller and not so well defined as they had been at 75 days (Fig. 4).

At 200 days after infection, tubercles of a number of animals on diet A were even smaller and less well defined than they had been in animals killed 20 days earlier. In others on this diet, however, the disease

apparently had been reactivated, as indicated by increased size of tubercles in the lungs, lymph nodes, and spleen, and by miliary tubercles in the lungs (Fig. 6).

Reactivation of the infection in animals on diet A was associated with chronic progressive glomerulosclerosis, which was recognized within 130 days after infection and progressed slowly thereafter. Thus, at 180 to 200 days the kidneys of at least half of these animals were deeply scarred and reduced in size. The more advanced stages of renal disease were accompanied by cardiac hypertrophy. Uninfected hamsters on diet A for an equal length of time did not develop disease of the kidneys.

Microscopic Appearances

From 75 to 130 Days after Infection (Figs. 7 and 8). Syrian hamsters that inhaled bacilli of the H37Rv strain developed characteristic tubercles within 28 days.[14] These were formed by monocytes that, with polymorphonuclear leukocytes in much smaller numbers, infiltrated and thickened the walls of alveoli and bronchioles and accumulated in compact masses within alveolar spaces. Thereafter the sharply outlined initial tubercles continued to expand without necrosis or other changes for about 80 days.

Secondary tubercles of the thoracic lymph nodes also were formed by monocytes that first appeared in tiny compact groups, scattered more or less at random in the nodes, about 35 days after infection.[14] Apparently these expanded slowly to fuse into larger masses by 130 days. At that time these lesions were infiltrated by small numbers of leukocytes. In the spleen, secondary lesions also arose as masses of monocytes that expanded slowly but were not often infiltrated by leukocytes until late in the disease. At first the secondaries in the liver were simply densely massed monocytes. Later these were mixed with lymphocytes and small giant cells.

Levels of dietary protein did not modify the histologic character of these lesions within 80 days, nor apparently influence the number of bacilli that might be demonstrated in them. At this stage of the disease bacilli were still found in sections as single cells scattered in the cytoplasm of monocytes.

Again at 125 to 130 days the inflammatory reaction was essentially the same in all diet groups. Now, however, alveolar walls within the central regions of initial tubercles had sometimes disappeared into the mass of monocytes. At this time, too, inflammatory cells (monocytes and polymorphonuclear leukocytes) were undergoing fragmentation within alveolar ducts and bronchioles at points within these central

zones. Leukocytes were most abundant in these foci of necrosis (Figs. 7 and 8). Peripheral to these relatively small central zones, by far the greater part of the initial tubercles still was formed as before. That is, the monocytes and polymorphonuclear leukocytes infiltrated the walls of alveoli and bronchioles and into alveolar spaces. The perivascular lymph channels also were filled by these cells, but the walls of the arteries and veins enclosed within the lesions usually were intact or, at most, had undergone slight thickening of the intima. At this time fibrosis was limited to the pleura where it adjoined tubercles in the lung parenchyma.

Between 80 and 130 days bacilli were not appreciably increased in the tubercles of animals on diet A (30 per cent protein). However, in animals on diet C (6 per cent protein) bacilli were found in much greater numbers in all lesions except those in the liver. In fact, bacilli were rarely demonstrated in the liver, even when they had become exceedingly numerous in the lungs, lymph nodes, and spleen.

From 130 to 200 Days after Infection (Figs. 9 to 14). Between 130 and 180 days after infection, tuberculosis of animals on diet C and of a majority on diet B (17 per cent protein) was characterized by increases in the number of bacilli, first in the central liquefying foci of initial tubercles, then throughout these lesions (Fig. 9). At the same time cavities were formed, apparently by expansion of the central necrotic foci of these tubercles, accompanied by dense infiltration of polymorphonuclear leukocytes, and more or less rapid endobronchial spread of the infection. However, cavities never came to occupy more than half of the initial tubercles even in animals on diet C, and usually were poorly defined spaces, partially filled and surrounded by inflammatory cells. Granulation tissue rarely formed about the cavities or anywhere within the tubercles of animals on diet C, except along the pleural surfaces, and even there but little collagen was produced.

Cavities were found occasionally in the initial tubercles of animals on diet A, evidence that necrosis had occurred here, too. In fact, a majority of the initial tubercles of this diet group undoubtedly underwent more or less central necrosis. But within 150 to 200 days the walls of cavities or the centers of the tubercles in this group had been transformed into pale-staining, eosinophilic "scars" suggesting amyloid, although amyloid could not be demonstrated. These scars contained little collagen, and a majority of the inflammatory cells within them were ill-defined (Fig. 10). Bacilli were difficult to demonstrate in any lesions of animals on diet A after 150 days.

Tubercles of the thoracic lymph nodes and spleens of animals on

diet C often were partially transformed into abscesses by 150 days after infection, i.e., they were densely infiltrated by polymorphonuclear leukocytes and more or less necrotic. In the later stages of the disease, bacilli became increasingly abundant in the lesions (Fig. 11). In animals on diet A, tubercles of the lymph nodes also formed abscesses that apparently persisted without great change from about 130 days after infection to the end of the observation period. At the same time, lesions in the spleens of animals on diet A usually were transformed largely into an eosinophilic substance (Fig. 12). Secondary tubercles developed to about the same degree in the livers of animals on all of the diets, but were largest and persisted longest in animals on diet C (Fig. 13). The livers of animals on diet A at 180 to 200 days often contained poorly defined foci of eosinophilic material that were believed to represent the "scars" of healed tuberculosis (Fig. 14). Amyloid could not be identified within these foci.

Tubercles of other organs of the body rarely developed to macroscopic size, and apparently did not contribute to the outcome of the disease.

TUBERCULOSIS INDUCED BY BACILLI OF LOW VIRULENCE (FIGS. 15 TO 20)

For the purposes of this study, virulence has been measured only by the capacity of bacilli of the H37Rv strain to induce tubercles to develop in the lungs of hamsters to diameters of about 5 mm. within 75 days. However, in one experiment with three diet groups of over 50 animals each, infections were unintentionally induced by bacilli of low virulence. Thus initial tubercles of these animals were less than half the expected size after 75 days, and even at 200 days were rarely more than 5 mm. in diameter.

The significant macroscopic features of tuberculosis induced by these bacilli of lower virulence are illustrated by Figures 15 to 20, which were prepared from animals on diets C (6 per cent protein) and A (30 per cent protein), killed at 75, 125, and 200 days after infection.

Levels of dietary protein apparently did not influence the susceptibility of hamsters to this strain of bacilli, for counts of initial tubercles after 75 and 125 days corresponded closely to the number of bacilli calculated to have been inhaled. Moreover, levels of dietary protein had not influenced the progress of the disease in these diet groups when the experiment was terminated 200 days after infection. Indeed, after developing for 200 days, the size and distribution of the lesions and their histologic appearances were about equal to those of lesions com-

monly found 75 days after infection by inhalation of bacilli of higher virulence. However, bacilli were much less frequent in all sites, and signs of necrosis were exceedingly uncommon in these lesions.

Later experiments with other hamsters of this stock and another culture of the H37Rv strain demonstrated that the results which have been described could not be attributed to a change in the capacity of the animals to acquire resistance. Therefore, they are presented as evidence of a change in virulence of the organism.

Discussion

In these experiments, levels of dietary protein neither altered the susceptibility of Syrian hamsters to inhalation infection by tubercle bacilli (H37Rv strain) of high or low virulence, nor modified the course of tuberculosis caused by organisms of higher virulence until the disease had progressed for upwards of 130 days. Beyond this time, however, tuberculosis of animals that inhaled the more highly virulent bacilli progressed most rapidly in animals on the lowest level of dietary protein. Tuberculosis induced by bacilli of low virulence progressed less rapidly in all diet groups than that induced by bacilli of high virulence. Still, its progress had not been modified appreciably by levels of dietary protein when the experiment was terminated 200 days after infection.

These observations are difficult to reconcile with the results of experiments on nutrition and tuberculosis induced in mice by injecting bacilli intravenously. Diets have modified this disease of mice within 4 weeks.[6,7] Perhaps a comparison of tuberculosis in different species may be impossible or, if not impossible, difficult when infections are induced by different routes and techniques, for these factors must determine, to a very great degree, the relations of hosts and parasites.[12] Thus, tuberculosis induced by inhalation differs widely in its pattern of development from that induced by injection.[14,15,21-25]

It is evident from recent publications that the implications of these differences have not been fully understood.[26] Therefore, a brief review may aid in orienting the results of the present experiments.

Tuberculosis induced by droplet nuclei infection, i.e., by inhalation, develops from separated bacilli deposited at isolated points on alveolar surfaces. Whether few or many bacilli are inhaled, development of a tubercle during the first 4 weeks passes through three distinct phases: (a) progressive growth of bacilli for about 2 weeks in alveolar macrophages that collect about the points at which bacilli have been deposited; (b) then acute inflammatory reactions in and about these

foci, with necrosis of alveolar macrophages and a sharp reduction in the number of bacilli; and (c) transformation of the inflammatory reaction into a less active process which, thereafter, is characteristic of the host-parasite combination (strain of bacillus and species of host animal[14,15]).

Whether few or many bacilli are inhaled, the acute inflammatory reaction 2 to 3 weeks after infection also causes organisms to be carried to the regional lymph nodes and beyond. But when infections are begun with a few initial foci in the lungs, the number of bacilli that are carried to the lymph nodes and beyond during the early stages of disease is always small and often is insignificant. Further spread of the disease within the lungs (and beyond in certain host-parasite combinations) may occur in time, but in all host-parasite combinations examined thus far, later progress of the disease can depend upon activity of a few (at times only one) initial tubercles in the lungs. Obviously, then, one virulent tubercle bacillus can be the infecting dose as well as the source of later progressive disease.

This pattern of tuberculosis (inhalation infection) has been demonstrated in mice, rats, guinea-pigs, hamsters, and rabbits. And by extrapolation, it can be applied readily enough to explain the common forms of pulmonary tuberculosis of infrahuman primates and wild carnivores.[27,28] Indeed, it applies quite as well to pulmonary tuberculosis of man. Therefore, it may not be dismissed as a unique phenomenon, reproducible only in experimental animals by special techniques.

Evidence is now at hand, and will be presented in detail later, to show that levels of dietary protein neither influence susceptibility of guinea-pigs or rats to infection by inhaled tubercle bacilli nor modify the earlier phases of this disease in these animals. Thus, the results of the present study on hamsters are not unique, and levels of dietary protein may be assumed to modify the course of tuberculosis only by modifying a capacity to acquire resistance.[16] However, changes in the pattern and progress of tuberculosis induced by organisms of high virulence became evident only after relatively prolonged intervals (about 130 days).

These observations are difficult to reconcile with current opinions on the nature of resistance to tuberculosis, which have attributed a dominant rôle to monocytes.[29] Indeed, monocytes are said to acquire the capacity to inhibit growth of virulent bacilli within 15 days after the animals had been injected with BCG.[30] Then, more recently, Lurie and associates[31] have presented evidence to show that monocytes of rabbits that had been inbred for resistance are endowed by heredity with a

greater capacity to inhibit multiplication of tubercle bacilli ($H_{37}Rv$ strain) than the monocytes of rabbits inbred for susceptibility. Even so, these "resistant" rabbits developed cavities and endobronchial tuberculosis within 8 weeks, while initial tubercles in the lungs of "susceptible" rabbits were still intact.

In part, this emphasis on the rôle of monocytes in resistance to tuberculosis may reflect the fallacies inherent in attempts to force the patterns of experimental disease induced by inoculation with large numbers of bacilli into correspondence with much more chronic processes. Also, these opinions may reflect the fallacies inherent in some experimental methods.[32,33]

The monocytes undoubtedly have important functions in resistance to tuberculosis, but apparently these functions depend for their development upon experience with infection. Indeed, highly uniform response to inhalation infection by virulent or by relatively avirulent bacilli is compelling evidence against the concept of native or hereditary resistance to tuberculosis.[11,13-15]

Levels of resistance, which are now measurable, develop rapidly when virulent bacilli are inhaled. Rabbits, for example, have resisted inhalation reinfection within 5 weeks after an initial inhalation infection by small numbers of virulent organisms.[11-13] Since the early stages of tuberculosis of rabbits and of hamsters are so closely similar, it may be assumed that hamsters also had developed equal levels of resistance within 5 weeks. But whether or not this is true, the ratio of bacilli to monocytes found in initial tubercles 5 weeks after the more highly virulent bacilli had been inhaled did not change appreciably during the following 6 weeks. Furthermore, in hamsters that had inhaled bacilli of low resistance, the ratio of bacilli to monocytes was established at a lower level, which did not change within 200 days. In either case, constant ratios of parasite to monocyte were established early and maintained for a time. The numerical value of these ratios and the intervals through which they have been maintained apparently depended upon the virulence of the parasite rather than the condition of the host and its level of dietary protein.

The relation of virulence to the immunizing properties of an organism is well known. And in these experiments the one essential difference between the manifestations of tuberculosis induced by organisms of different virulence has been the response to levels of dietary protein. This has been associated with a wide difference in the apparent rate of growth of the organisms even during the earlier phases of the disease. Therefore we suggest that resistance to tuberculosis in the hamster is

developed through at least two steps. The first occurs within the third week after inhaled infection, irrespective of the host and virulence of the parasite.[13,15,34] This endows the monocytes with a capacity to limit, more or less, the growth of bacilli, depending on their virulence. The second step develops much more gradually as the bacilli growing in the expanding tubercles stimulate antibody formation. This occurs most readily when protein is supplied in adequate amounts and the bacilli grow at rates adequate to supply the needed antigens, but not to overwhelm the animal.

Failure of resistance in animals on lower levels of dietary protein may be interpreted to mean that the antigens also can operate to reverse and abolish the first level of resistance. This has occurred most readily when the intake of protein was low and the liver had been badly scarred by tubercles. It has occurred also with higher levels of protein when glomerulosclerosis was far advanced, although the effects of renal damage on tuberculosis were then much less striking.

The first step in developing resistance to tuberculosis in these animals may be attributed directly to a change in the monocytes. The second step demands another mechanism, for now it must protect against the growth of bacilli in extracellular foci as well as within the monocytes. It is in this second stage that dietary protein has been found to be a critical factor in determining the level of resistance.

Summary and Conclusions

Groups of young adult Syrian hamsters were allowed at least 6 weeks for adjusting to experimental diets, after which they were caused to inhale small numbers of tubercle bacilli of human origin and maintained on these diets until killed for study at stated intervals between 75 and 200 days after infection. Three isocaloric diets were used. These supplied approximately 30, 17, and 6 per cent protein.

Levels of dietary protein did not influence susceptibility to inhalation infection by tubercle bacilli of high or low virulence, nor modify the progress of disease induced by organisms of low virulence within 200 days. Moreover, levels of dietary protein did not modify the progress of tuberculosis induced by organisms of high virulence until about 130 days after infection. Beyond this time, however, progress of tuberculosis was most rapid in animals on 6 per cent dietary protein. In hamsters on 30 per cent protein, tuberculosis was arrested, or regressed for a time. Its reactivation in animals on this diet was associated with progressive glomerulosclerosis.

It is concluded that, in hamsters, dietary protein can be a critical

factor in the development of effective levels of resistance to tuberculosis. This requires relatively prolonged interaction of host and virulent parasites, and apparently reflects the combined actions of the monocytes and other factors which, presumably, are antibodies. Rapid failure of resistance in association with hepatic and renal disease supports this suggestion.

It is concluded, also, that the highly uniform reaction of these animals for upwards of 130 days, irrespective of their diet, is further evidence against the concept of native or hereditary resistance to tuberculosis.

We are indebted to Mr. William Fore, Department of Pathology, University of Pennsylvania, for the photographs.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

Figures 1 to 14 show lesions of tuberculosis induced by bacilli of high virulence.

Figures 1 to 6. Macroscopic lesions in thoracic lymph nodes and one lung of each animal, and in spleens of representative animals. The thoracic lymph nodes have been cut vertically to the long axis of the body. Usually the left lung, which is not divided into lobes, is shown.

FIG. 1. Litters of 4 and 6 animals on diet C (6 per cent protein) 75 days after infection.

FIG. 2. Random animals on diet A (30 per cent protein) 75 days after infection. Secondary tubercles in the lungs are less numerous than in Figure 1, but are more numerous in the spleens. Variation in the number of secondary tubercles in lungs is greater among litter mates of Figure 1 than among random animals of Figure 2.

FIG. 3. One litter of 7 animals on diet C, 150 days after infection. Lungs and lymph nodes show wide variations in the progress of tuberculosis; the lesions in the spleens are more uniform.

FIG. 4. Random animals on diet A, 180 days after infection; little evidence of secondary spread beyond the lymph nodes.

FIG. 5. One litter of 7 animals on diet C, 180 days after infection; endobronchial and splenic tuberculosis is conspicuous.

FIG. 6. Random animals on diet A, 200 days after infection; progressive disease, usually miliary and associated with glomerulosclerosis.

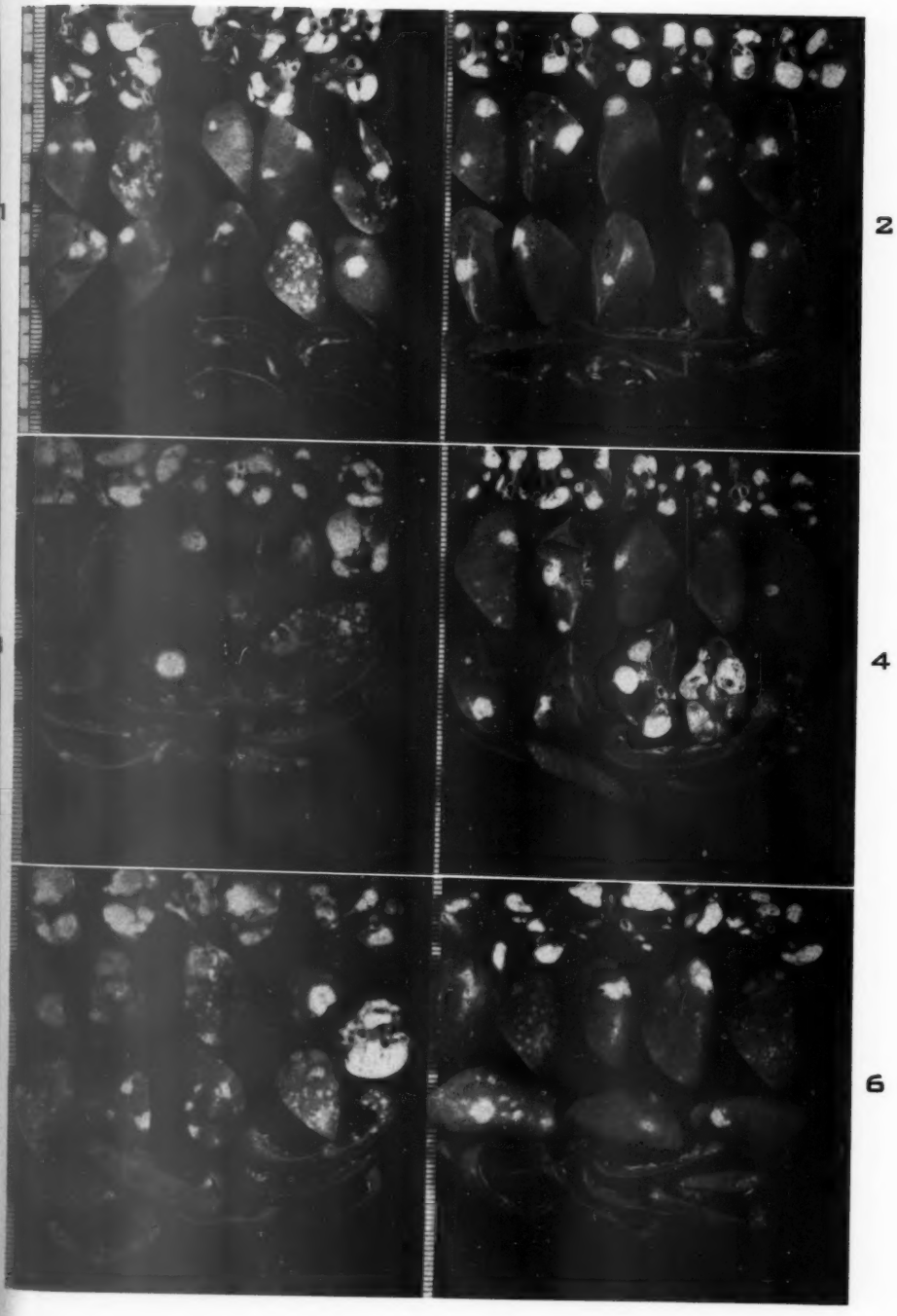
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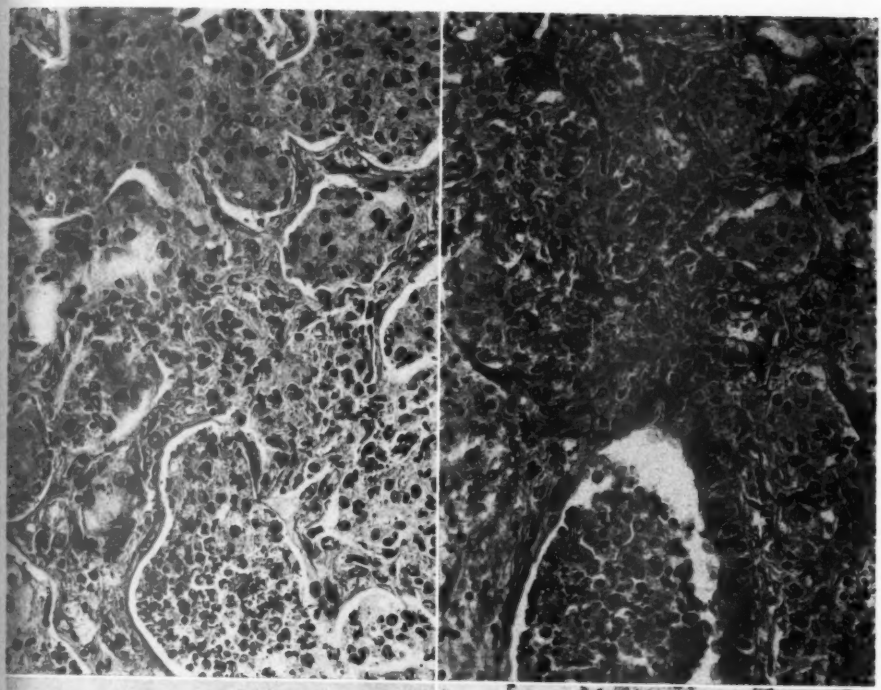
FIG. 7. The central zone of an initial tubercle from an animal on diet C, 125 days after infection. Alveolar walls are thickened and alveolar spaces and ducts are filled by monocytes and polymorphonuclear leukocytes with necrosis beginning in an alveolar duct. Hematoxylin and eosin stain. × 350.

FIG. 8. The central zone of an initial tubercle from an animal on diet A, 125 days after infection, with exudate in a terminal bronchiole. Hematoxylin and eosin stain. × 350.

FIG. 9. Bacilli in cells about the border of a cavity from an animal on diet C, 180 days after infection. The cavity was partially lined with epithelium. Carbol fuchsin-light green stain. × 800.

FIG. 10. Border of a cavity from an animal on diet A, 200 days after infection. Hyalinized tissue forms the wall of this cavity; a few bacilli are present. Carbol fuchsin-light green stain. × 800.



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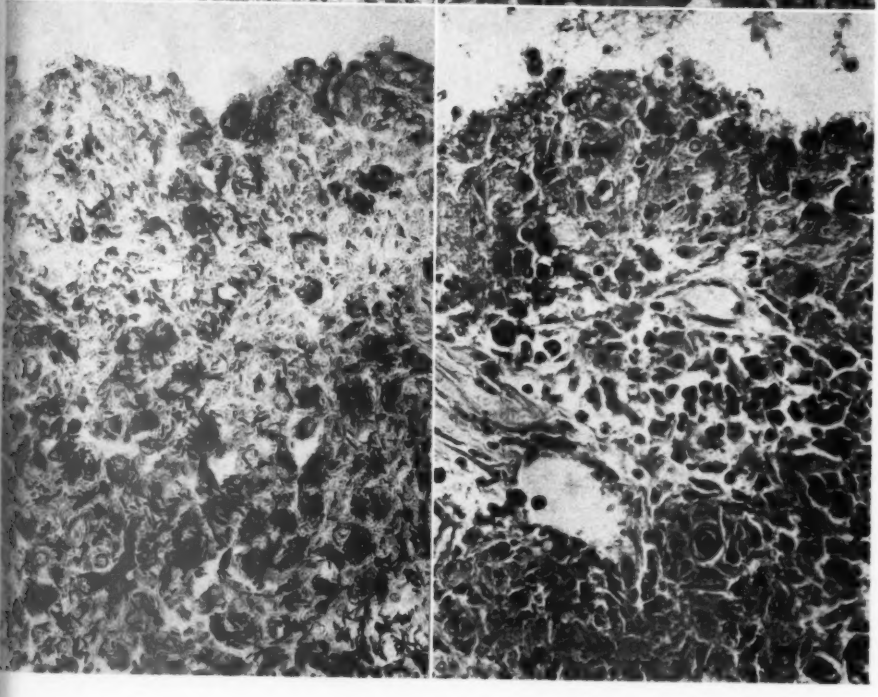

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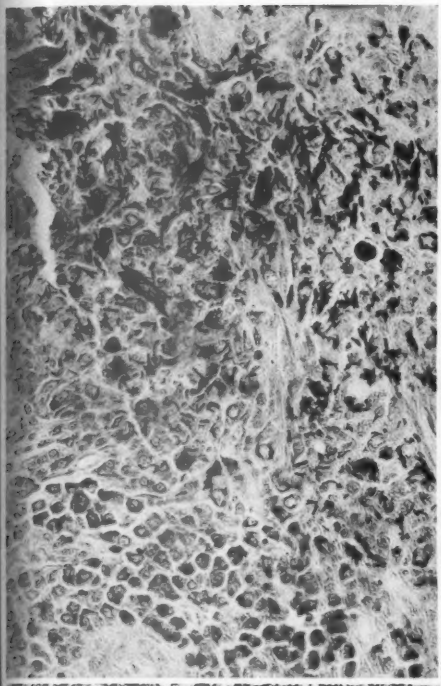
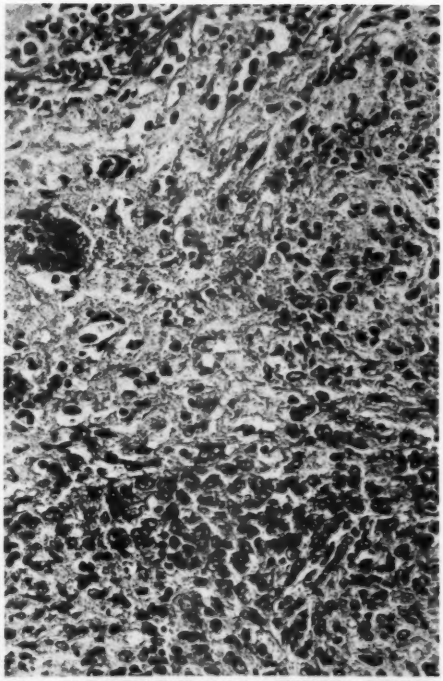
FIG. 11. Bacilli in a splenic tubercle of an animal on diet C, 180 days after infection. Carbol fuchsin-light green stain. × 800.

FIG. 12. Hyalinized tissue in the spleen of an animal on diet A, 200 days after infection. Bacilli are not demonstrated. Hematoxylin and eosin stain. × 800.

FIG. 13. Tubercles at the periphery of a liver lobule of an animal on diet C, 155 days after infection. Hematoxylin and eosin stain. × 250.

FIG. 14. Hyalinized tissue near the periphery of a liver lobule of an animal on diet A, 200 days after infection. Hematoxylin and eosin stain. × 250.





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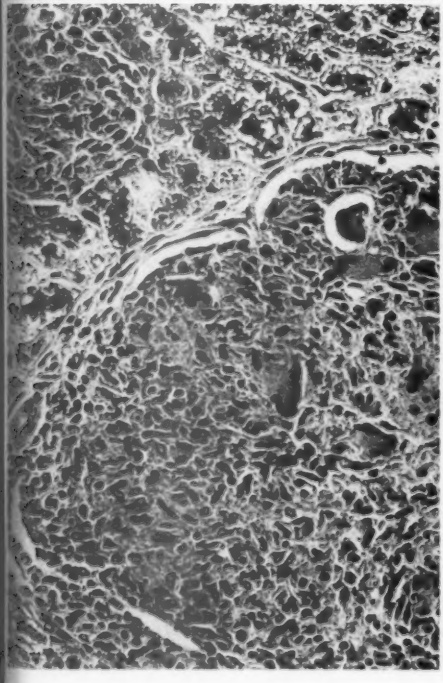

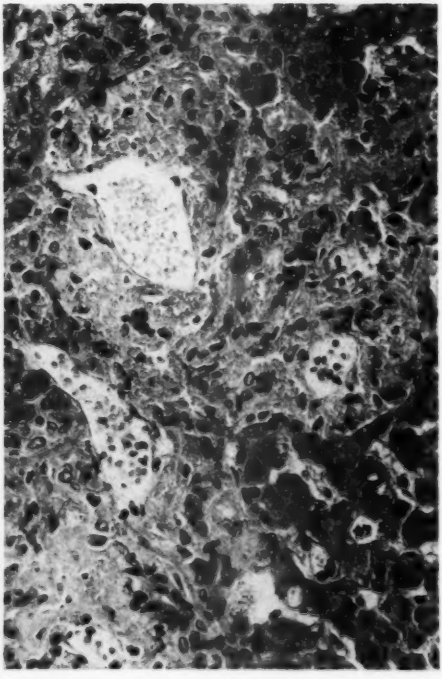

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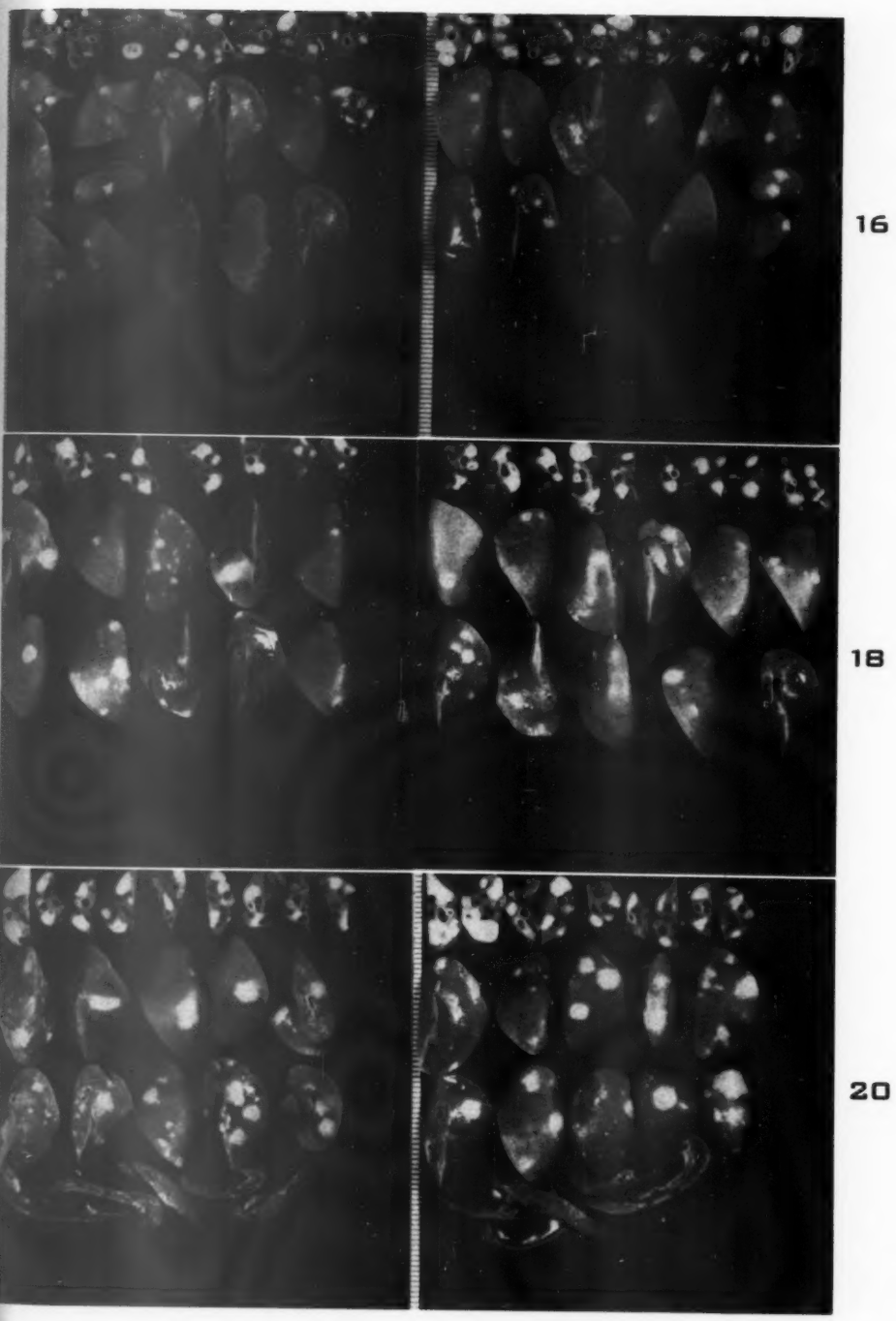
Figures 15 to 20 are from lesions of tuberculosis induced by bacilli of low virulence.

Figures 15, 17, and 19 illustrate tuberculosis in animals on diet C (6 per cent protein); Figures 16, 18, and 20, tuberculosis in animals on diet A (30 per cent protein).

FIGS. 15 and 16. Tubercles of the lungs and thoracic lymph nodes of random animals, 75 days after infection. Spleens are not involved; for comparison with Figures 1 and 2.

FIGS. 17 and 18. Tubercles in lungs and thoracic lymph nodes of random animals. 125 days after infection. Spleens are not involved.

FIGS. 19 and 20. Tubercles in lungs, thoracic lymph nodes, and spleens of random animals, 200 days after infection.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STUDIES OF THE EFFECT OF THE GENERALIZED SHWARTZMAN REACTION ON THE LESIONS OF EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS *

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Four major categories of demyelinative diseases have been described in man. These are multiple sclerosis, Schilder's diffuse periaxial encephalitis, post-infectious and post-vaccinal encephalomyelitis, and acute necrotizing hemorrhagic leuko-encephalitis.[1] The histologic features which characterize these diseases as a group can be reproduced readily in experimental animals by inoculation with nervous tissue and adjuvants. The resulting experimental disease is known as experimental allergic encephalomyelitis (EAE) (reviewed by Hurst[2] and Innes[3]). Focal perivenous collections of inflammatory cells, occurring most frequently in the white matter and associated with myelin breakdown but with relatively little damage of axis cylinders, are found both in the demyelinative diseases and in EAE. Efforts to reproduce the special features which distinguish the individual categories of demyelinative disease have, however, met with limited success. The pathologic changes characteristic of post-vaccinal encephalomyelitis have been well duplicated by EAE in various animal species. In experimental animals with chronic EAE, lesions that resemble those of multiple sclerosis have been found by several workers.[4-6] Schilder's disease has not been reproduced successfully in animals nor has acute hemorrhagic necrotizing leuko-encephalitis, though there are reports of hemorrhagic lesions in unusually acute or intense EAE in the monkey[7] and of necrotizing lesions in the dog.[8]

The present report summarizes the results of a series of experimental attempts to produce lesions comparable to those of acute necrotizing hemorrhagic encephalomyelitis in rabbits. It was thought that the characteristic lesions of EAE might be modified so as to resemble more closely those of this human disease. For this purpose the hemorrhagic diathesis characteristic of the general Shwartzman response[9,10] was produced in rabbits with EAE, and the resulting lesions were compared with those produced by the same treatment in normal rabbits and with the lesions of uncomplicated EAE.

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Methods

Production of EAE. Animals in which EAE was to be studied received a single injection in one or more toe pads of an antigen mixture containing 2 parts of nervous tissue (rabbit cord, bovine white matter, or certain fractions of bovine white matter) mixed by grinding in a mortar with 3 parts of oil (Bayol F) containing 3 mg. per ml. of heat-killed tubercle bacilli. A number of these rabbits had been employed in other studies in our laboratory.[11] All developed typical EAE between the 9th and 25th day after inoculation. Details of the clinical and histologic evaluation of this disease may be found in our earlier publications.[11,12]

Production of the Generalized Shwartzman Reaction. Three samples of meningococcal toxin were used to produce the generalized Shwartzman reaction. Two, generously provided by Dr. Gregory Shwartzman and labelled T3347 Mg44B and T3351 Mg44B, will be referred to as toxins A and C. Dr. Lewis Thomas kindly supplied another sample which will be designated toxin B. Each was used over a period of approximately 9 months following its receipt. Fresh dilutions were prepared for use each week. All animals were injected intravenously with appropriate volumes of toxin in either a 1:50 or a 1:500 dilution. Thorotrast, a stabilized 24 to 26 per cent suspension of thorium dioxide, was obtained from the Hayden Chemical Corporation, New York (lots no. 215 and 217) and from Testagar and Co., Inc., Detroit (batch no. 06166). A standard dose of 3 ml. per kg. was employed. Animals were given either a single dose of toxin, two successive doses of toxin, or thorotrast followed by toxin. The significance of these different procedures is considered in detail in papers by Thomas and his coworkers.[13,14]

Albino female rabbits of a mixed stock, weighing 2.0 to 3.5 kg. and obtained from local dealers, were used. Table I summarizes the num-

Table I

Summary of Toxins and Dosage

	Rabbits with EAE				Normal controls			
Toxin	1 dose	2 doses	1 dose following thorotrast	Total	1 dose	2 doses	1 dose following thorotrast	Total
A	14	13	10	37	0	9	3	12
B	2	3	4	9	1	3	2	6
C	3	11	3	17	3	5	5	13
Total	19	27	17	63	4	17	10	31

bers of rabbits subjected to each of the indicated procedures with each sample of toxin in the present study. The actual doses used varied widely (between 0.0004 and 0.04 ml. of undiluted toxin) and seemed to bear little relation to the lethal effects of the toxins or their capacity to produce such changes as were observed. Normal rabbits which received comparable injections of toxin and thorotrast (Table I) were regarded as a suitable control group for the study of lesions of the nervous system in the generalized Shwartzman reaction. A second control group consisted of numerous rabbits with EAE studied in other connections and not given toxin or thorotrast. A few animals received toxin shortly before the anticipated onset of EAE. As this procedure involved considerable guesswork, it was not extended.

Observations. The animals were examined frequently after the onset of EAE and the injections of thorotrast or toxin. Survivors were sacrificed and necropsies were performed approximately 24 or 48 hours following the last toxin injection. A cisternal puncture was done at this time; the total protein of the cerebrospinal fluid was determined nephelometrically after precipitation with sulfosalicylic acid,[15] and the white cells were counted. Histologic study was made of the kidney, liver, spleen, lungs, and heart, and of sections of cervical, thoracic, and lumbar spinal cord with accompanying roots and ganglia. In addition, in many animals the brain stem, cerebellum, cerebral cortex, optic nerves, and sciatic nerves were examined also.

Results

Rabbits with EAE injected with Shwartzman toxin showed, in a few instances, a clear-cut exacerbation of their neurologic symptoms. More commonly, however, they exhibited apathy and general weakness, ruffled fur, gasping respiration, and opisthotonus, signs described by Thomas and Good[10,13,14] in their reports on the general Shwartzman reaction. Occasionally, within a few hours hemorrhages were seen in the eye or in the subconjunctival tissues, and bloody urine might be passed. These latter signs were observed also in the normal controls injected with toxin. Death occurred in about one half the experimental animals with EAE (30 of 63) within 24 hours after the last toxin injection, whereas only one fourth of the controls succumbed to the lethal effect of the toxin (7 of 31).

In those animals which survived the injections of toxin, cisternal puncture was performed at the time of sacrifice. With satisfactory taps, median values of 58 mg. per cent total protein and 73 cells were obtained in 14 cerebrospinal fluids from EAE animals given toxin and

30 mg. per cent protein and 5 cells in the normal controls which received toxin. When no toxin was given, values of 39 mg. per cent protein and 10 cells were found in rabbits with mild EAE, 222 mg. per cent protein and 96 cells in rabbits with severe EAE, and 25 mg. per cent protein and 5 cells in normal animals.[16] The cells were almost entirely lymphocytes, but in one or two fluids appreciable numbers of polymorphonuclear cells were found. Unfortunately, few good specimens were obtained in animals which later proved to have extensive Shwartzman lesions in the nervous system—nearly all such animals either gave bloody fluids or died before a tap could be carried out. Of two successful taps in such animals, one fluid contained large numbers of polymorphonuclear leukocytes and gave a total count of over 2,000 cells; while the other contained 18 cells, all lymphocytes. A striking finding was that even when the technique of cisternal puncture was quite satisfactory, about one half of the fluids were bloody, both in the EAE group (11 of 28) and in the controls (9 of 14). The slightly lesser incidence of bloody fluids in the rabbits with EAE may result from the higher frequency of early death in this group—taps were not performed on animals which had been dead for more than a few minutes and therefore no fluid was obtained from many of the most severely affected animals. The incidence of bloody fluids in a series of EAE animals not given toxin was considerably less (18 of 68), and in animals without disease, still less (8 of 72).

Histologic examination of the central and peripheral nervous systems of animals given toxin showed the presence of easily demonstrable changes. Widespread and intense congestion of small vessels was invariably present. Small thrombi and vascular plugs of polymorphonuclear leukocytes and platelets also were seen in many of the animals subjected to any of the Shwartzman procedures. In many congested vessels, the outlines of the erythrocytes were indistinct with the result that the content of these vessels had a homogeneous appearance not unlike that of a thrombus (Fig. 12). However, by proper adjustment of the light, it was possible to distinguish conglutinated red cells from true thrombi. Other vessels were obstructed by amorphous, slightly eosinophilic or slightly basophilic material containing no cells whatsoever. In animals which received thorotrast, this material, which was easily recognized by its refractile and quasi-crystalline appearance, was seen in small vessels and capillaries throughout the nervous system, but particularly in the meninges. It was present in the thrombi already described, and in animals which survived for any length of time it was present in considerable quantities in macrophages in and around vessels.

Frequently, necrotic foci were found in the central nervous system of animals with EAE which had received toxin; these foci surrounded the plugged vessels and usually contained extensive infiltrations of polymorphonuclear cells (Figs. 9 and 10). There appeared to be a continuous gradation from lesions with only a few leukocytes among the histiocytic infiltrates characteristic of EAE, usually around veins or capillaries blocked by leukocyte thrombi, to abscesses several hundred microns in diameter. Sometimes a necrotic focus was of sufficient size to involve a considerable area of the spinal cord. Such lesions appeared to arise by extension from smaller foci or from the confluence of several small lesions (Figs. 1 to 4). When a single cross section of the spinal cord was severely affected, i.e., contained five or six lesions of moderate size, microscopic examination usually showed that otherwise typical EAE lesions contained thrombosed capillaries and an unusual number of polymorphonuclear cells. The meningeal exudate also contained many polymorphonuclear cells in some areas. A characteristic feature of this change was its rather focal character; there were many regions of the nervous system not showing the change at all, while others were heavily involved.

Hemorrhage was a particularly frequent finding in rabbits given toxin. It appeared in several distinct forms. Hemorrhage in the subarachnoid space, in the interstices of the choroid plexuses, or in the ventricles was found in animals which had received toxin with or without EAE. The presence of hemorrhage of this type on post-mortem examination did not appear to be related to the finding of apparent subarachnoid hemorrhages on cisternal puncture; one must assume, therefore, that the latter were "bloody taps" due to the transfixion of congested vessels. Petechial hemorrhage about congested vessels in the central gray matter of the spinal cord (Fig. 11) was common also in both the test and control groups given toxin. Similarly, hemorrhage was found frequently in the epineurium of peripheral nerve in both groups. A less frequent finding was massive hemorrhage in the parenchyma of the cord, roots, or ganglia (Figs. 5 to 8), sometimes superimposed on necrotizing lesions but often occurring in the absence of change of other types. That the bleeding in lesions of this type was present as an *ante-mortem* event appeared to be established by the inflammatory cells in the hemorrhagic areas and occasionally by fibrin in and around involved vessels. Where these vessels could be identified, they appeared to be small veins.

Neither the necrotic nor the hemorrhagic lesion occurred in the parenchyma of the peripheral nerves, though a number of the specimens examined contained the characteristic focal lesions of EAE.[16] How-

ever, too few nerves were examined, sixteen altogether, to make this finding certain.

The relation of these fairly characteristic changes to the presence of EAE lesions in the central nervous system is brought out in Table II. Lesions of all of the types described in the preceding paragraphs occurred much more commonly in rabbits with EAE given toxin than

TABLE II

Incidence of Lesions of Shwartzman Type in the Central Nervous Systems in Various Experimental Groups

Type of treatment	Number of rabbits	Number with lesions of Shwartzman type	Lesions in central nervous system					Number with Shwartzman lesions*
			Thrombi	Necrotic foci	Hemorrhage			
					Subarachnoid	Petechial in gray matter	Massive in parenchyma	
EAE and Shwartzman	63	28	22	9	15	7	10	15
Shwartzman only	31	6	2	0	3	2	0	0
EAE only	92	10	0	2	4	4	0	2

*Includes only necrotic lesions and/or massive hemorrhage in the parenchyma of the nervous system.

in either of the control groups. However, since there may be many *ante-mortem* and *post-mortem* causes for petechial or subarachnoid hemorrhage and since hemorrhages of this type were seen in animals from both control groups, it is difficult to evaluate their significance in relation to either EAE or the Shwartzman procedure. The thrombi, as will be indicated in the discussion, were regarded as part of the Shwartzman mechanism. Lesions comparable to the classical Shwartzman reaction, with massive hemorrhage and necrosis, were produced in 15 of the 63 animals in which the Shwartzman procedure was superimposed on pre-existing EAE (cf. summary of findings in Table III). The injection procedure influenced the pathologic picture produced, all three toxin samples having similar effects. The focal necrotic lesion occurred commonly after large single doses of toxin, whereas massive hemorrhage, with or without necrosis, usually was seen following two toxin injections and more frequently following thorotrast and toxin. Though severe lesions were observed with widely different doses of toxin, it is apparent that smaller doses were effective when two injections of toxin were employed and still smaller doses were active following thorotrast injection. The length of the animal's survival did not seem to affect the character of the lesions (all animals died or were killed within 48 hours).

TABLE III

TABLE III

Visceral Involvement in Rabbits with EAE and Shwartzman Lesions in the Central Nervous System

Rabbit no.	Dose and lot of toxin (ml./kg.)	Survival (hours)	CNS lesions* T	CNS lesions* N	CNS lesions* H	Kidney N	Kidney H	Liver N	Liver H	Spleen N	Spleen H	Heart N	Heart H	Lung N	Lung H
Single toxin dose															
47	0.03A	5	+	**+**	0	0	0	+	0	0	++	±	0	0	±
407	0.02A	7	+	**++**	0	0	0	+	0	±	±	0	0	0	0
410	0.03A	7	++	**+++**	0	+	+	+	+	0	0	+	0	+	+
73	0.03A	16†	+++	+	0										
3	0.01A	17†	0	+	**++**										
Two toxin doses (24-hour interval)															
2	0.01A	2†	+	0	**+**	0	0	+	0	++	++	0	+	0	+
7	0.01A	24	0	0	**+**										
90	0.006A	<18†	+	0	+	++	+++	+	±	±	++	0	0	0	+
94	0.007A	20	0	0	**+**	0	+	++	+	+	0	±	0	0	0
611	0.002A	1†	0	++	++	0	0	+	+	0	+	±	+	+	+
661	0.0008B	31	+	**+++**	**+++**	0	0	0	±			0	0	0	0
Thorotrast ‡ and toxin (3 to 4-hour interval)															
4	0.001A	12†	++	+	**+++**	+	+	0	+	0	+	+	++	0	++
96	0.0014A	12†	+	0	**++**	0	0	±	0	+++	+	±	±	0	++
656	0.0005B	48	+	++	+++	0	±	+	±	++	±	+	±	0	0
478	0.0015C	12†	++	++	**++**	0	+	0	0	++	+++	0	0	+	++

T = Thrombi, with or without polymorphonuclear cells.
N = Necrotic foci.
H = Massive parenchymal hemorrhage.

* Reactions shown in boldface type occurred in clear-cut relation to EAE lesions.
† Spontaneous death.
‡ Thorotrast: 3 ml./kg.

Histologic study suggested that focal and necrotizing lesions were always related to the pre-existent lesions of EAE. They either involved veins already affected by that disease or appeared at the edge of more extensive infiltrations of mononuclear cells. Hemorrhage, on the other hand, often involved apparently normal tissue, and in these cases it was difficult to ascertain whether the bleeding vessel was one affected by EAE. The severity of EAE did not influence the appearance of Shwartzman lesions in the nervous system, i.e., moderate or severe lesions of Shwartzman type occurred to about the same extent and with about the same frequency in animals with different degrees of EAE. However, it seemed that there might be an optimum period during the development of EAE for the production of Shwartzman lesions; such lesions were not produced when toxin was injected a day or two before the anticipated onset of EAE (5 of the 63 test animals were treated in this way) or more than 4 days after the onset (11 of the 63 animals).

The histologic examination of the major viscera (kidney, liver, spleen, heart, lung) revealed the presence of lesions comparable to those observed in the central nervous system; i.e., congestion and thrombosis, focal and diffuse necrosis, and hemorrhage. The necrotic areas usually were invaded by large numbers of polymorphonuclear cells. The spleen and, to a lesser degree, the lung were more frequently affected than the other major organs and usually more severely. All of the viscera were not involved in every animal; in many rabbits lesions were found in only one or two organs, the rest remaining normal. In the two groups given toxin, necrotic foci or hemorrhages were found in one or more organs in about two thirds of all animals. The incidence of severe lesions in individual organs was of the order of 10 to 30 per cent. The kidneys were involved in 9 of 78 animals (11.5 per cent), a figure agreeing closely with that of 10 per cent found by Smith and Thomas[17] for older rabbits. There were somewhat fewer lesions in EAE animals given toxin than in normal controls given toxin, but this finding may have depended on the earlier death of the rabbits in this group. No changes of the Shwartzman type were observed in viscera of rabbits with EAE not given toxin, constituting the large control series.

The visceral findings in the 15 animals with typical Shwartzman lesions of the central nervous system are summarized in Table III. A comparison with other rabbits indicates that the major organs in these 15 animals were involved to the same degree as in the experimental group as a whole. The same lack of parallelism is seen between involvement of the central nervous system and that of the other organs (type and degree) as among the organs themselves.

Discussion

In rabbits subjected to various procedures which are effective in producing the hemorrhagic diathesis known as the general Shwartzman reaction, lesions of a type not previously described in the central nervous system of that species were found. Congestion, often accompanied by subarachnoid hemorrhages and by small hemorrhages by diapedesis in the central gray matter of the spinal cord, and thrombi of amorphous material resembling fibrinoid and of leukocytes and platelets were seen in the central nervous system of both the experimental group with EAE given toxin and of normal animals given toxin, though somewhat more frequently in the former. Lesions of this type were not seen in animals which had not received toxin. Full-blown hemorrhagic and necrotic lesions in the central nervous system occurred only in animals with EAE given toxin and with a frequency comparable to that of similar lesions in other organs.

Earlier attempts to produce local Shwartzman reactions in the nervous system had proved to be unsuccessful,[9,18] though Bock[19] reported paralysis in rabbits given a preparatory dose of toxin intracerebrally and a subsequent provocative dose intravenously. No thorough investigation of the effects of the general Shwartzman reaction on the central nervous system has been reported previously. The brief papers of Marburg and Shwartzman[20] and Adant *et al.*[21] do not make possible an evaluation of the nervous lesions in this experimental situation. In the earlier series of studies of Thomas and Good,[10,13,14] the nervous system apparently was not examined. More recently, Brunson *et al.*[22] reported the finding of fibrinoid-like material in the vessels of the choroid plexus and the cerebral vessels, in one instance adjacent to an area of acute encephalomalacia.

In the present study, the production of typical Shwartzman lesions in the central nervous system appeared to depend on the establishment by means of toxin of the basic Shwartzman change in animals whose nervous system was in some way predisposed through the presence of another disease, namely EAE. The exceptional occurrence of similar lesions in animals with EAE not given toxin possibly may be attributed to intercurrent infection accompanied by a Shwartzman mechanism. Typical changes were notably absent from the nervous systems of normal animals given toxin.

The lesions of the general Shwartzman reaction were observed also in the kidneys, livers, spleens, hearts, and lungs of animals given large single doses of toxin, two doses of toxin, or thorotrast followed by toxin. They were not seen in a large control series to which no toxin

was administered. The histologic details of the lesions in each organ are not considered in the present paper, as they have been amply described by others. The general character of the visceral lesions and of the lesions in the nervous system was clearly the same, though the various organs were involved to different degrees in individual animals. The incidence of lesions in any one organ (including the nervous system) was low, about 10 to 30 per cent. This low incidence of disease in our animals is in agreement with the findings of Smith and Thomas,[17] who reported that only about 10 per cent of mature rabbits develop typical lesions in the kidney and that susceptibility to a given dose of toxin is relatively unpredictable. Unfortunately, EAE cannot be produced in younger animals,[12] which are more uniformly susceptible to the general Shwartzman reaction and less susceptible to the lethal effect of toxin injection and which might, therefore, have developed more frequent and perhaps more striking nervous lesions.

The mechanism whereby the observed lesions of the nervous system are produced cannot differ fundamentally from the mechanism acting elsewhere in the body to produce similar lesions. Stetson[23] has stressed the importance of leukocyte-platelet thrombi occluding damaged vessels in the genesis of the local Shwartzman phenomenon in the skin. Kane, Good, and Thomas[24] have drawn attention to the importance in some of the viscera, notably the kidney, of vascular occlusion by a homogeneous material which resembles fibrinoid in conventional histologic preparations. In our experiments, the nervous lesions appeared to depend in part on local vascular damage, probably resulting from the presence of EAE, and in part on general vascular occlusion by both leukocyte-platelet thrombi and the fibrinoid-like material already mentioned. Since injections of the toxin did not produce Shwartzman lesions in the central nervous system of normal animals, the normal vasculature in this tissue must be insusceptible to the preparatory action of toxin, differing in this respect from that in other organs. The basis of such insusceptibility and its relation to other well known peculiarities of the central nervous system such as the blood-brain barrier and the special cerebrospinal fluid circulation remain entirely unknown. EAE may cause a breakdown of the blood-brain barrier.[25] It has been suggested that EAE is an allergic reaction of the tuberculin type[12]; and tuberculin reactions have been shown to act as prepared sites for Shwartzman responses.[26] The local predisposing effect of EAE appeared to exist only during the first 4 days of the disease. This period is comparable to the duration of preparedness of 2 to 4 days following a single toxin injection. There may, indeed, have been more than one

mechanism operating in these experiments, as there appeared to be qualitative differences between the lesions resulting from a single toxin injection and those produced by two toxin injections or thorotrast and toxin.

The lesions produced in the nervous system in the present experiment differ in a number of particulars from the lesions of hemorrhagic, necrotizing leuko-encephalitis in man. In the latter,[27] the principal histologic changes are necrosis of small veins and of brain tissue around these vessels, with fibrin exudation, multiple punctate hemorrhages, and an intense inflammatory reaction in the damaged brain tissue, the Virchow-Robin spaces, and the meninges. Thrombosis by fibrin, platelets, and polymorphonuclear leukocytes is not prominent, although seen in a few vessels, while fibrin impregnation of the vessel wall and the adjacent tissue and large infiltrations of polymorphonuclear leukocytes are the dominant histologic changes in acute and rapidly fatal cases. In the experimental lesion under consideration, in contrast, thrombosis is one of the main features. The necrotic and hemorrhagic changes are not coextensive with the EAE lesion but rather appear to involve limited areas or individual vessels in the affected region. Hemorrhage, when it occurs, is massive rather than punctate. Finally, the involved region of the central nervous system is, of course, that most affected by EAE in the rabbit, namely, the spinal cord, meninges, posterior roots, and spinal ganglia rather than the brain and brain stem, which have been the main site of acute necrotizing hemorrhagic leuko-encephalitis in man.

Actually, there have been three other approaches to the problem of inducing, by immunologic means, experimental lesions resembling those of acute necrotizing, hemorrhagic leuko-encephalitis in man.

(1) Arthus reactions have been induced in the brain by the introduction of foreign antigen directly into the brain substance of sensitive animals.[28-30] The techniques have varied in a number of particulars. The resulting lesions (in rabbit or monkey) consist of the same elements as the well known Arthus reaction in the skin: central necrosis with massive hemorrhages, congestion and thrombosis, the accumulation around vessels in or near the necrotic area of hematogenous elements and, if time allows, of gitter cells, and edema and cellular infiltration of the adjacent meninges. Occasionally, diffuse hemorrhage is observed. These lesions differ from the human disease by the prominence of thrombosis, the localization to the region of the injected antigen, and, in many animals, by the diffuse character of the hemorrhage.

(2) The so-called Forssman or carotid syndrome in guinea-pigs is

produced by injecting into the common carotid artery an antibody against the Forssman antigen (widespread in guinea-pig tissues, probably largely in the walls of blood vessels).[81] This experimental lesion consists of diffuse vascular dilatation and the later appearance of scattered perivascular foci of softening (demyelination accompanied by destruction of nerve and glia cells and loss of axis cylinders). There is neither hemorrhage nor thrombosis, but a pronounced mononuclear cell response occurs. This lesion also fails to duplicate in detail the changes in the human disease.

(3) The lesions of EAE itself may vary considerably. Thus, in the rabbit very severe, early lesions may contain considerable numbers of polymorphonuclear leukocytes; and, in rare instances, as indicated in our control group with EAE that had been given no toxin, there may be necrosis or mild hemorrhage. In some species, the more severe lesions may be accompanied by hemorrhage, necrosis of vessel walls, their impregnation with fibrin, and the presence of large numbers of polymorphonuclear cells in the perivascular and meningeal lesions. The dog and monkey in particular show this change.[7,8] The lesions in these species are thus similar in distribution and character to those in the human disease which we are considering. It appears, therefore, that the presence of hemorrhage and necrosis and the exudation of polymorphonuclear cells may be merely the expression of the intensity of the response, which in milder reactions consists almost entirely of perivascular histiocyte activation and demyelination. Support for this notion is gained by considering not only the pathologic similarities but also the clinical course in each instance. Cases of typical disseminated encephalomyelitis may follow by several days nondescript prodromal (often respiratory) illnesses exactly as do the most severe hemorrhagic cases. Perhaps, then, it is wisest to focus attention on the intensified disease occurring in individuals prone to develop unusually intense sensitivity rather than on other types of sensitivity or more complex mechanisms.

Summary

Sixty-three rabbits with experimental allergic encephalomyelitis (EAE) were given intravenous injections of meningococcus toxin in dosages suitable for the production of the generalized Shwartzman response. Thirty-one normal rabbits similarly injected and 92 rabbits with EAE but not injected with toxin served as control groups. Toxin was injected once, twice with a 24-hour interval, or once 4 hours after intravenous thorotrast.

Almost half of the experimental rabbits showed changes in the cen-

tral nervous system attributable to the injections of toxin. Fifteen of the 63 showed full-blown Shwartzman lesions, with hemorrhage and necrosis of the nervous parenchyma, usually superimposed on the pre-existing lesions of EAE. Such hemorrhagic or necrotic lesions were not observed in the control group given toxin alone and occurred in only 2 of the 92 EAE rabbits not given toxin. There was no correspondence between the occurrence of lesions in the central nervous system and the presence or severity of Shwartzman lesions in the major viscera. Typical lesions were found only in rabbits injected with toxin during the 4 days following the onset of EAE.

The lethal effect of toxin injection was enhanced by the presence of EAE. Bloody spinal fluids were found in about half of the experimental group and of the control group receiving toxin. Meningeal hemorrhage and petechial hemorrhages in the gray matter also were found in both groups.

The histopathologic features of the Shwartzman lesion in the nervous system are compared with the changes in hemorrhagic, necrotizing leuko-encephalitis of man and with the lesions produced experimentally by other immunologic techniques.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIGS. 1 to 4. Necrotic and hemorrhagic lesions of spinal cord. Rabbit 32-661, 4 plus EAE of 4 days' duration. Two doses of toxin A (0.0008 ml. per kg.) 24 hours apart. Killed 31 hours after second dose. Bloody spinal tap. Figures 2, 3, and 4 are enlargements of necrotic, hemorrhagic areas in the upper cord section shown in Figure 1. Hematoxylin and eosin stain. Figure 1, × 7; Figures 2 and 3, × 90; Figure 4, × 340.

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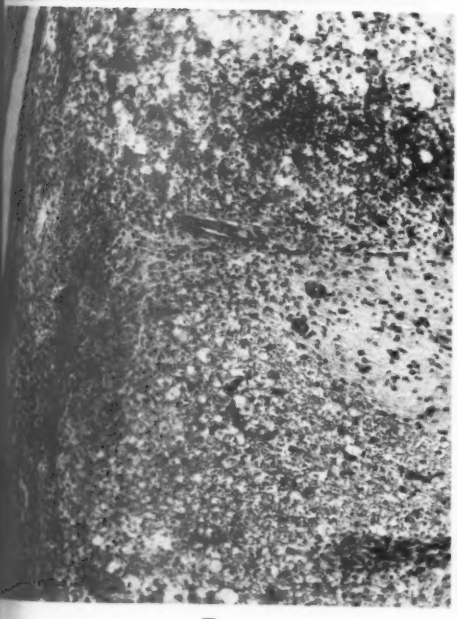

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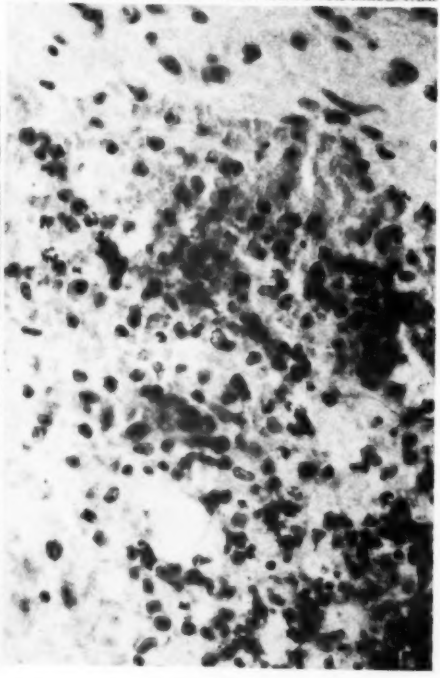

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FIGS. 5 to 8. Hemorrhagic lesion of spinal root, ganglion, and meninges. Rabbit 29-4, 3 plus EAE of 2 days' duration. Thorotrast followed by toxin A (0.001 ml. per kg.). Died approximately 12 hours following toxin administration. Figure 6 illustrates root hemorrhage. Figure 7 shows hemorrhage superimposed on EAE lesion in ganglion. Arrow indicates mass of polymorphonuclear cells, probably forming a cellular thrombus in a small vessel. An enlargement of the same area is shown in Figure 8. Hematoxylin and eosin stain. Figure 5, × 7; Figures 6 and 8, × 320; Figure 7, × 90.

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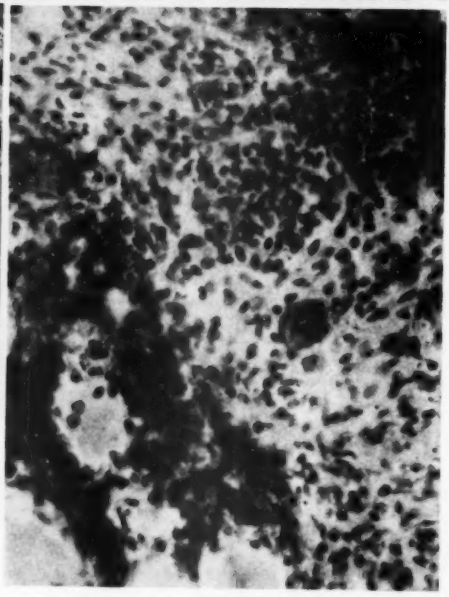

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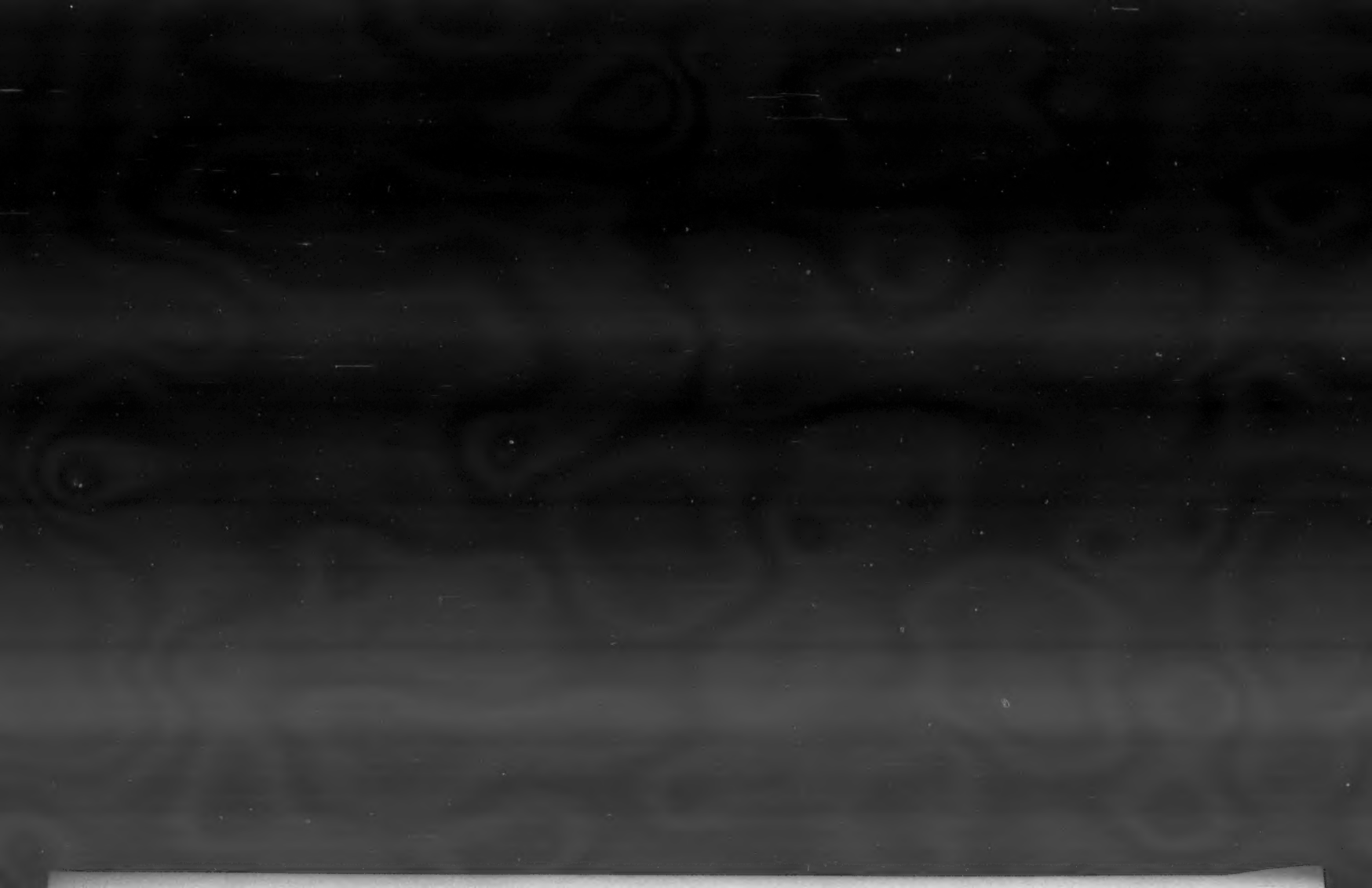
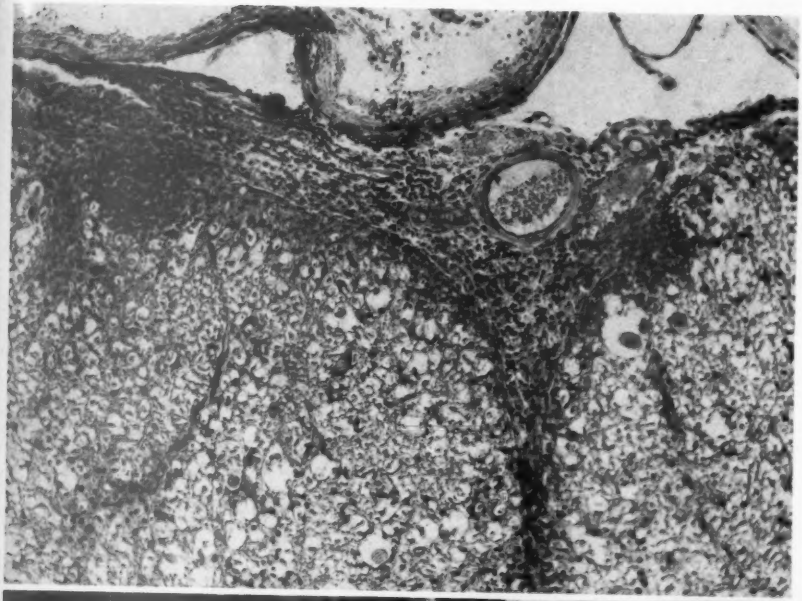
FIGS. 9 and 10. Necrotic foci with massive polymorphonuclear invasion in area with extensive EAE. Rabbit 29-410, 4 plus EAE. Single dose of toxin A (0.03 ml. per kg.) on first day of symptoms. Killed 7 hours later. Hematoxylin and eosin stain. Figure 9, × 125; Figure 10, × 320.





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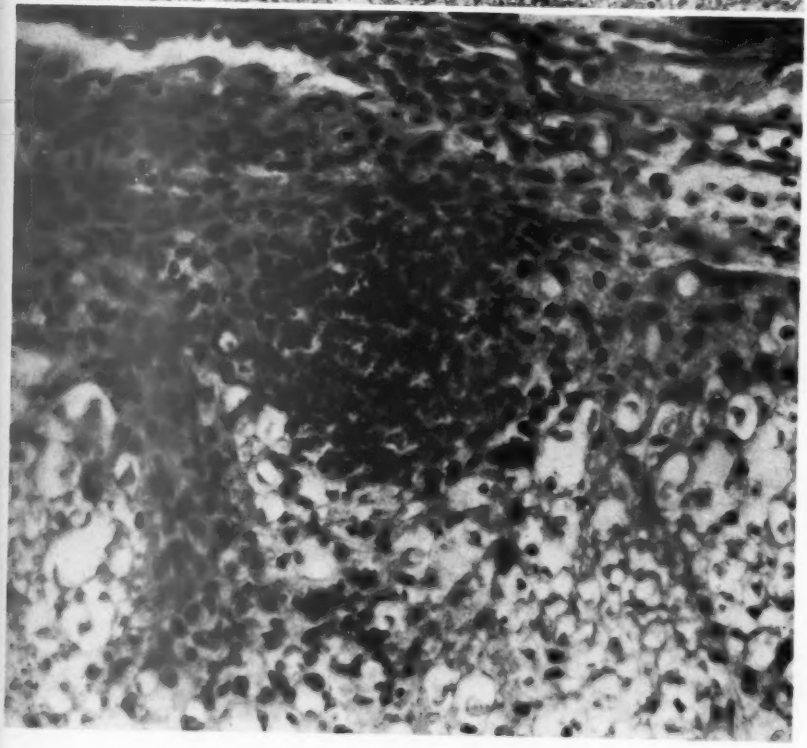

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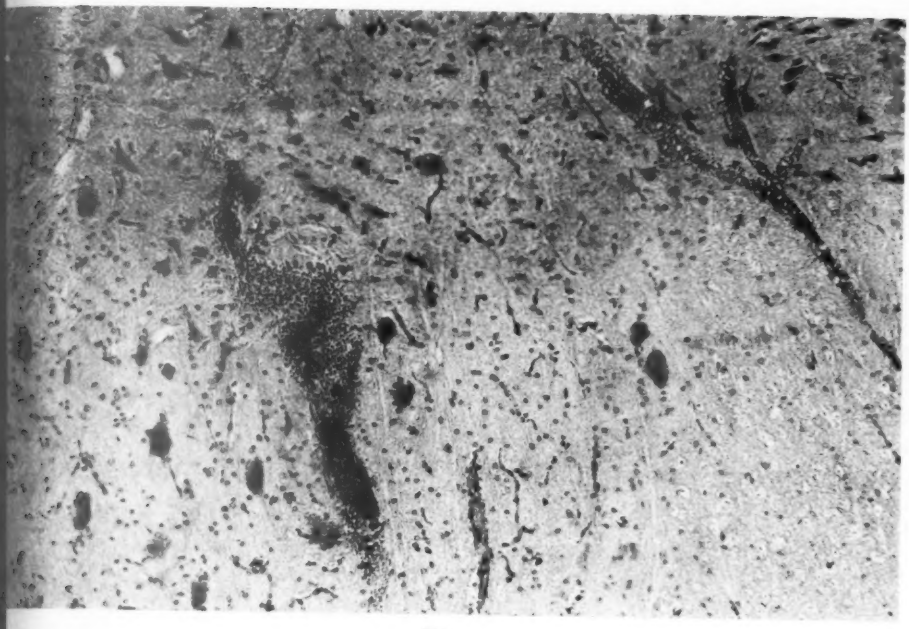
FIG. 11. Congestion and hemorrhage in gray matter of spinal cord remote from EAE lesion. Rabbit 29-7, 3 plus EAE of 2 days' duration. Two doses of toxin A (0.01 ml. per kg.) 24 hours apart. Killed 24 hours after second dose. Hematoxylin and eosin stain. × 115.

FIG. 12. Congested and thrombosed vessels in periphery of spinal cord. Vessels filled and distended by fibrinoid material. Rabbit 29-73, 2 plus EAE. Single dose of toxin A (0.03 ml. per kg.) on first day of symptoms. Died approximately 16 hours later. Hematoxylin and eosin stain. × 415.



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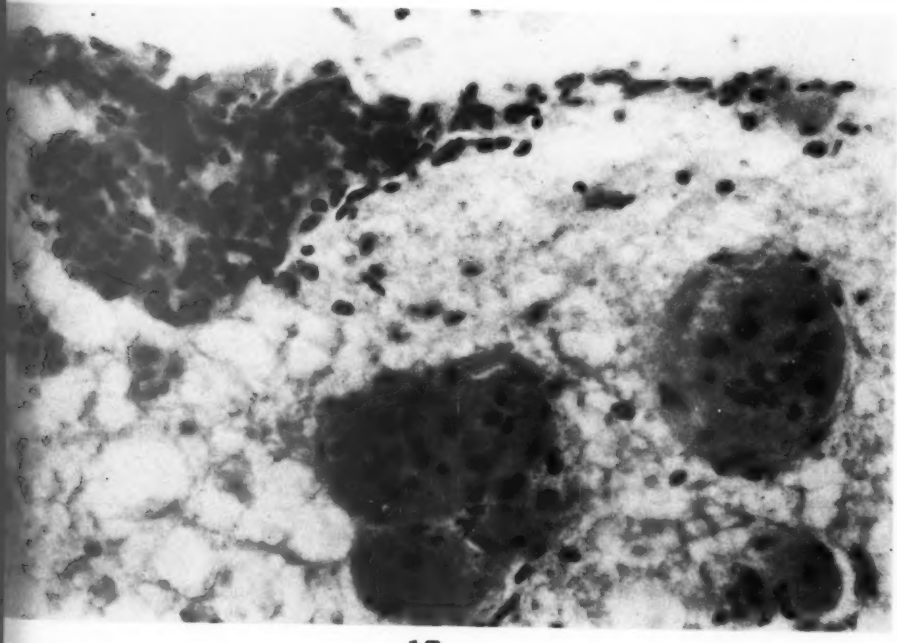

12

ANTERIOR PITUITARY GLANDS IN PATIENTS TREATED WITH CORTISONE AND CORTICOTROPIN *

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In a notable contribution to knowledge of the pathology of Cushing's syndrome, Crooke,[1] in 1935, described a characteristic alteration in the cytoplasm of the pituitary basophil cells. In each of his original 12 cases he observed a replacement of the basophilic granules by a homogeneous substance of similar staining qualities, a process which he termed hyaline change. The almost constant appearance of this phenomenon received early confirmation[2-6] and it became of importance as establishing a common morphologic denominator for widely divergent etiologic concepts.

In the initial phases the hyaline material was noted either in the peripheral areas of the cells, or, more frequently, in a crescent midway between the nucleus and the cell membrane. Eventually the entire cytoplasm became hyalinized. In contrast to this remarkable cytoplasmic transformation, the nuclei evidenced no stigmas of degeneration. Other authors soon described nuclear ballooning, nucleolar hypertrophy, multinucleation, and generalized enlargement of the basophils.[7-9] Severinghaus[10] described cytoplasmic degranulation and perinuclear blistering vesiculation in the pituitary basophils of the case of Cushing's syndrome on which data were given by Graef and associates.[11] However, the perinuclear changes were stressed in this single observation, and the hyaline changes which were present were not emphasized.

McLetchie[8] pointed out the coexistence of normal granularity and the encroaching hyaline material, and elaborated on the increased incidence of abnormal vacuolization within these cells. Transitional basophils and large amphophilic cells with foamy cytoplasm, large vesicular nuclei, and ill-defined cell borders were thought by Gellerstedt[7] and by Mellgren[9] to represent a phase in the evolution of hyalinized cells.

Although Crooke[12] has denied the common occurrence of hyaline

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basophils in states of adrenal cortical hyperfunction other than Cushing's syndrome, some investigators have described these cells in cases of adrenal virilism[5,13-15] and virilizing ovarian tumor.[16]

Hyalinized basophils have been observed infrequently in a variety of non-endocrine diseases and in normal persons. Crooke found minimal hyaline changes in only 9 of 350 non-Cushing pituitary bodies.[1] Ecker[17] discovered that only 8 of 721 pituitary glands of patients not afflicted with Cushing's syndrome contained hyalinized basophils, and emphasized the normal size of these cells and the lack of nuclear enlargement and multinucleation. Mellgren[5] regarded a small number of hyaline cells as normal, with varying increases in several conditions, Cushing's syndrome and virilizing syndromes exhibiting the greater numbers.

Crooke[1] expressed the belief that this hyaline change represents "an expression of altered physiological activity" and concluded that it "is the alteration of fundamental significance in the causation of the syndrome." This view was not inconsistent with Cushing's concepts of etiology[18] based on pituitary hyperfunction, and acquired some adherents.[5,7,8] Two schools were thus established, the first led by Crooke, the second or adrenogenic school included Bauer,[19] Albright,[20] and Kepler and associates.[21,22]

The latter investigators were early critics of the hypothesis of a pituitary origin of the syndrome, and Kepler developed the thesis that the hyaline changes are a result rather than the cause of certain hyperadrenal states. He pointed out the paucity of the change in basophil adenomas,[22] and the apparent cure by subtotal adrenalectomy of some cases of Cushing's syndrome.[21] He commented that hyaline cytoplasm implies a retrograde if not a degenerative change.[22]

Despite the accumulation of extensive data, the etiology of Cushing's syndrome has not yet been fully elucidated. For the cases in which the condition has been successfully treated in recent years, the therapy in the great majority has consisted of surgical removal of hyperfunctioning adrenal cortices,[21-23] while a lesser number have responded to roentgen irradiation of the pituitary gland.[24,25]

The recent widespread use of corticotropin (ACTH) and 17-hydroxy-11-dehydro-corticosterone (cortisone) in various non-endocrine diseases has frequently resulted in the production of hyperadrenal states indistinguishable from spontaneous Cushing's syndrome.[26,27] The occurrence of hyaline changes in the pituitary basophils of these hormone-treated patients would be telling evidence against the hypothesis that such changes are indicative of pituitary hyperfunction. Such

was suspected when Golden and associates[28] examined the hypophyses of two patients with chronic glomerulonephritis who received corticotropin prior to death. The first patient, who demonstrated "a distinct increase in basophilic cells, which displayed striking hyaline cytoplasmic changes as described by Crooke" had received 400 mg. of corticotropin intramuscularly during a 5-day period before death. The second patient was given 490 mg. of corticotropin in 8 days, treatment terminating 7 days before death. Hyaline material was present in a few scattered basophilic cells. Statistically significant basophilia occurred in these cases at the expense of the chromophobes, Rasmussen's differential counting method being used. The pituitary body of a third patient with myasthenia gravis who had received two courses of corticotropin of 975 mg. and 500 mg., respectively, 9 months and 6 months before death, was examined. The paucity of Crooke's cells in this pituitary gland suggested reversibility of the phenomenon.

In the pituitary body of a patient with Cushing's syndrome who survived 66 days after subtotal adrenalectomy, Kepler[29] recorded that 40 per cent of the extra-adenomatous basophils were hyalinized. Luft[24] found only small numbers of Crooke's cells in a patient with Cushing's syndrome who committed suicide almost 2 years after considerable improvement had followed pituitary irradiation. Luft stated that the predominant pathologic cell in this case was the hypertrophic amphophil of Gellerstedt[7] and Mellgren.[5]

Laqueur's[30] study of 8 patients treated with corticotropin, cortisone, or both substances confirmed the induction of hyaline changes in the basophils but indicated no correlation between the degree of change and the time and amount of hormone employed. No alterations were noted in one patient with acute leukemia who received 7.35 gm. of cortisone in 35 days. Advanced hyalinized basophils with hypertrophic nuclei were found by Gilbert-Dreyfus and Zara[31] in the pituitary body of a female patient with disseminated lupus erythematosus who had received 10 gm. of cortisone in 2 months.

Long before the advent of purified corticotropin and adrenal steroid preparations, Severinghaus and Thompson,[32] by prolonged injection of extracts of sheep's anterior pituitary gland, had produced basophil alterations in dogs which strongly resembled Crooke's changes. Organization of the basophilic granules into spherical masses and the eventual formation of a basophilic amorphous colloid-like substance had been described. Later, Heinbecker[33] described almost identical changes in the basophils of dogs whose supra-optic and paraventricular nuclei of the hypothalamus had been destroyed. These alterations

closely resemble the early cytoplasmic changes in the basophils which we will describe. By the use of corticotropin and cortisone, degranulation of the pituitary basophils has been induced experimentally in dogs, guinea-pigs,[34] and rats.[35]

On the basis of the foregoing observations, it is reasonable to suppose that the hyaline change of Crooke is a result of the exposure of pituitary basophils to excessive amounts of 11-oxygenated adrenal steroids. Because of the small number of cases reported and the apparent similarity, if not identity, between these induced hyaline cells and the Crooke cells of Cushing's syndrome, this study was undertaken.

Material and Methods

The pituitary glands of 76 patients who had received therapeutic cortisone, corticotropin, or both substances before death were studied. Following standard fixation in 10 per cent formaldehyde and imbedding in paraffin, four or more midhorizontal sections were made. Although Crooke and Russell's modification[36] of Mallory's acid-fuchsin aniline-blue method is tinctorially good and widely employed, there is great variation in the times of washing and differentiating for different pituitary bodies. By reversing the order of application of the acid and basic dyes, that is, staining first with aniline blue, then with acid-fuchsin, these difficulties were minimized.

The clear gray-blue refractile appearance of the hyaline cytoplasm contrasts well with the deep blue basophilic granules and closely resembles the results obtained with Crooke's stain. The Mallory-Heidenhain stain is not recommended when hyaline material is sought. Probably because no hematoxylin is used, there is very little color contrast between inconspicuous hyaline areas and the adjacent cyanophilic granules.

For greater clarity in presentation, the cases are divided into three major groups. Group I includes cases in which cortisone alone was used. Group II comprises cases in which corticotropin alone was used. In the cases of group III both substances were used. Since arbitrary classification of the degree of pituitary change is impossible, brief descriptions of the principal changes are given. Differential cell counts were not done. The great variation in the distribution of basophils throughout the pituitary gland does not permit valid predictions of their relative numbers from a few sections without complete counts. Much contradiction exists regarding the presence of excessive numbers of basophils in spontaneous hypercortico-adrenal states. Certainly, basophilia has not been established in Cushing's syndrome and adrenal

virilism,[1,5,6,14] although this is at variance with Pearse's recent study[15] wherein increased numbers of "mucoid" cells were found. The basophilia in the cases examined by Golden and associates[28] seems to indicate that such a change can occur early in the course of corticotropin therapy.

Group I. Cases in Which Cortisone Alone Was Used

Considered in group I are 49 cases in which cortisone alone was used. Histologic examination of the hypophyses confirmed the recent studies demonstrating Crooke's changes in patients treated with cortisone. For comparison, a normal pituitary gland is shown in Figure 1. There appeared to be a rough correlation between the degree of cytologic change and the amount of steroid administered, and it seemed possible to define a distinct evolution of changes in the basophils. In the description of cytoplasmic changes which follows, the terms colloid and hyaline are used to designate the physical characteristics of the cytoplasm of the basophils.

No abnormalities could be detected in those cases in which only 100 to 350 mg. of cortisone had been given during the day or two before death. Distinct atrophy and agglutination of the basophilic granules appeared in patients who had received a few hundred milligrams of the steroid in the several days preceding death. A ground-glass or colloid appearance best describes the deeply cyanophilic degranulated cytoplasm of this stage, although in a few cases a finely reticulated cytoplasm was noted. These phases of degranulation are best represented by 3 cases. In the first, 400 mg. of cortisone was given in the 4 days preceding death from acute lymphatic leukemia (Fig. 2). In the second, almost 2 weeks elapsed between the final administration of 450 mg. in 3 days and death from atypical encephalitis. In the third case, myocardial infarction and multiple cerebral abscesses were found at necropsy 10 days after treatment with 400 mg. of cortisone in 3 days. Although this prehyaline form of degranulation dominated the cytologic change in these hypophyses, degranulated cells were observed also along with hyalinized cells in cases in which larger and more protracted doses had been given. The coexistence of different stages of change in the basophils was prominent in a fifth case of this group, in which 2.4 gm. of cortisone had been given over a 26-day period concluded 6 days before death (Fig. 3). Nuclear changes usually were absent in the cells showing early alterations, and vacuolization varied greatly, but usually exceeded the normal amount.

Next in the evolution of hyalinization was the appearance of discrete, hyalin-like masses. These masses frequently were observed as thin

peripheral rims or as irregular clumps throughout the cytoplasm. In the cells containing the peripheral hyaline rim, the colloid or ground-glass alteration was characteristic of the remaining cytoplasm. At times, fibrillary strands of homogeneous hyaline material were present, with an apparent distortion of the cell outline. At this stage, ballooning of the nuclei with a prominent nucleolus was evident. Two cases of the cortisone group typify this cytoplasmic change. The first patient was given 2 gm. of cortisone in two 2-week courses, beginning, respectively, 90 days and 2 weeks before death (Fig. 4). The second patient succumbed to acute lupus erythematosus after administration of 5.8 gm. of cortisone in 124 days, terminating 8 days before death (Fig. 5).

Increasing formation of hyaline material was the next apparent alteration. It appeared that there was progressive enlargement of a peripheral rim of hyaline material, although no characteristic point of origin was apparent. The completely hyalinized cells were, as a rule, only slightly enlarged and many were of normal diameter. Multiple nuclei were rare in hyalinized cells, but were much in evidence in certain larger cells containing prehyaline cytoplasm. Typical of hyaline change was the pituitary body of a girl. This 5½-year-old child had acute lymphatic leukemia and received 4.2 gm. of cortisone in 33 days before death (Figs. 6 and 7). For comparison with Figure 7, the Crooke cell of spontaneous Cushing's syndrome is shown in Figure 8.

Prominent acidophilic nucleoli frequently were encountered within the ballooned nuclei in the cases displaying increasing hyalinization of cytoplasm. The most striking instances of nucleolar enlargement were found in the larger basophils containing a degranulated, colloid-appearing cytoplasm. In some cells the nucleolus occupied the entire nuclear volume. Multinucleation was noted frequently in these large basophils, three or four nuclei being the maximum.

No consistency was evident in the degree or type of vacuolization of the altered basophils. Widespread extreme vacuolization to the point of cell distortion was seen in patients treated briefly as well as in those given prolonged hormone therapy. However, cells with early cytoplasmic changes tended to display vacuolization, while hyalinized cells contained few or no vacuoles.

Minimal or no vacuolization and virtual absence of nuclear and nucleolar changes were characteristic of the 15 children in the series, whose ages ranged from 5½ months to 10 years. Regardless of the type of hormone administered, cytoplasmic degranulation was the only prominent change. In those receiving moderate to substantial amounts of cortisone, corticotropin, or both substances, a much more uniform

and widespread degree of hyalinization was evident than occurred in the adults.

Although there was some evidence of correlation between the degree of cellular change and the duration and amount of hormone dosage, many exceptions were encountered. Small to large numbers of normal appearing basophils were seen in several cases in which substantial amounts of cortisone had been used, ranging from 2.4 gm. in 26 days to 35.4 gm. in 300 days. Outstanding resistance to hyalinization was observed in a patient who had received an average of 120 mg. of cortisone per day for 285 days, a total dose of 34.2 gm., for rheumatoid arthritis. Many of the signs of Cushing's syndrome appeared before death. Examination of the pituitary body in this case revealed surprisingly few hyalinized cells, and of these none were completely hyalinized. Agglutinated granules of marked cyanophilic quality, arranged in a coarsely reticulated pattern, dominated the histologic picture (Fig. 9).

In 4 cases considerable periods had elapsed between cessation of therapy and death. Fewer than 20 per cent of the basophils contained small rims of hyaline cytoplasm in the first case, in which 1.5 gm. of cortisone had been administered for bronchial asthma 5 months before necropsy. No basophilic abnormality could be detected in the second case, in which death from myasthenia gravis occurred 3 months after the administration of 2 gm. of cortisone. In the third instance, examination of the pituitary body of a rheumatoid arthritic patient, 65 years of age, who had died 9 months after administration of 5 gm. of cortisone, revealed some partially hyalinized basophils interspersed with many normal cells. An extremely rare hyaline remnant was the sole pituitary abnormality in the fourth case, in which death from myasthenia gravis occurred 2 years after therapy with 1.8 gm. of cortisone.

Group II. Cases in Which Corticotropin Alone Was Used

Corticotropin was the sole hormonal therapy in the 15 cases of group II. The cytologic alterations in this group were similar to those described in the cases in which cortisone alone was used. Since corticotropin causes excessive production of adrenocortical steroids,[27] it seems probable that cortisone-like steroids were responsible for the transformation of the basophils.

On the basis of clinical experience, it has been estimated that by weight corticotropin is approximately twice as potent therapeutically as cortisone.[37] The small number of cases in this group permits no definite conclusion as to the relative potency of these hormones in

inducing hyaline change. However, the average total amount of cortisone administered in 49 cases was 4.9 gm., while the average amount of corticotropin in 15 cases was 855 mg. The largest amount of corticotropin, 2.8 gm., was given over a 46-day period to a child, 5 years old, who succumbed to acute lymphatic leukemia. Advanced hyaline changes were present. Extensive hyaline changes were observed also in the pituitary body of a child, 3 years of age, who received 1.7 gm. of corticotropin in 135 days for the nephrotic syndrome. Twelve days was the longest period between cessation of therapy and necropsy, and typical widespread alterations were seen. These observations suggest that corticotropin is more effective than cortisone, weight for weight, in the induction of hyaline changes.

Group III. Cases in Which Both Cortisone and Corticotropin Were Used

In 12 cases both corticotropin and cortisone were used (group III). No appreciable difference in the basophilic response from that already described was apparent.

Correlation of Cytologic and Clinical Data

The cytologic changes in the foregoing groups were all confined to the basophil cells. Although cellular atrophy and the occurrence of small, densely staining nuclei in the acidophils have been described in the pituitary glands of patients with Cushing's syndrome and virilizing syndromes,[6,8] we found no changes in the acidophils.

Pronounced clinical hypercorticoidism developed in 11 of the 76 cases, the manifestations varying from rapid gain of weight with acneiform rash, facial rounding, or both, in 7 cases, to a fully developed picture of Cushing's syndrome in 4. Among 9 adult patients who were treated solely with cortisone, the smallest amount given was 3.7 gm. in 39 days, or an average daily dose of 95 mg. Another patient received 35.4 gm. of cortisone in 300 days, the average daily dose being 117 mg. The combined adrenal weights in this case were 5.6 gm. For the entire group receiving only cortisone, the average total amount was 14.4 gm. in 154 days, the average daily dose being 93.5 mg. In addition to the cortisone-treated patients, 2 patients received substantial amounts of corticotropin and in one case administration of 1.7 gm. of corticotropin resulted in a pronounced "Cushing-like" syndrome.

Although alterations in the basophils were striking and advanced in these patients with clinical hypercorticoidism, the extreme changes that occur in some cases of spontaneous Cushing's syndrome were not

observed. While the hyalinized cytoplasm attained the clear gray, refractile nature seen in Crooke's cells, the numerous hypertrophic, completely hyalinized cells with ballooned nuclei commonly observed in cases of long-standing Cushing's syndrome were not encountered. We believe, therefore, that it is appropriate to emphasize degranulation and prehyaline change in describing the development of the hyaline cell. It is not surprising that such advanced changes were not encountered, since the longest period of treatment was only 10 months, while Cushing's syndrome usually develops in a more insidious and prolonged fashion.

Comment

The similarity between the pituitary histopathologic picture of Cushing's syndrome and that of the cases comprising this study group is readily apparent. The hormonal imbalances which characterize the latter probably are similar to those which give rise to spontaneous Cushing's syndrome.[27]

In practically all verified cases of Cushing's syndrome examined at necropsy, hyalinized basophils have been found in the non-adenomatous portion of the anterior pituitary body. Approximately one half of these glands contain basophilic adenomas,[24,29] an incidence considerably higher than the 6.1 per cent reported by Costello[38] in 1,000 unselected necropsies. Hyperplasia of a regenerative type in response to the injurious effect of high levels of circulating steroids has been proposed to account for this increased incidence.[29] If this is true, it would appear that both the hyalinized basophils and the basophilic adenomas are consequences of adrenal cortical hyperfunction. However, since roentgen irradiation of the pituitary body occasionally brings about a temporary or permanent remission of Cushing's syndrome,[24,25] a pituitary origin of the disorder remains a possibility. Nevertheless, the regular production of Crooke's changes in the pituitary bodies examined in this study strongly supports the thesis that these cells represent a retrograde phenomenon rather than the causative lesion. Because only two to four tissue sections were studied in some of the cases of this series, we cannot comment on the incidence of basophilic adenomas.

The apparent transition of the basophils in this study from an early phase of colloid or "ground-glass" degranulation, reminiscent of the canine basophil alterations described by Severinghaus and Thompson[32] and by Heinbecker,[33] through hypertrophy of the cell and its nuclear elements, to the presence of varying amounts of hyaline cytoplasmic material, agrees rather closely with the extensive reports of Gellerstedt,[7] Mellgren,[9] and Luft.[24] It is somewhat at variance with the

hyaline replacement of normal granular cytoplasm described by Crooke[1] and by McLetchie[8] in cases of spontaneous hyperfunction of the adrenal cortex. The Scandinavian group[7,9,24] and Burt[39] have noted, in addition to the typical Crooke's cells, varying numbers of hypertrophic amphophilic cells with indistinct cell borders, foamy or finely granulated cytoplasm, and large distended polymorphic nuclei sometimes containing tremendously enlarged acidophilic nucleoli. Similar cells were noted in the case of Graef and associates[11]; however, the fuchsinophilic masses were identified as pyknotic nuclei. Possibly owing to differences in staining methods, we did not observe any cells fulfilling all of these criteria. Rather, we observed a very large basophil with distinct cell membrane containing a deeply basophilic degranulated material and large distended nuclei, frequently multiple and often replaced by a giant acidophilic nucleolus. Mellgren[9] has expressed the belief that the hypertrophic amphophil represents a transitional stage in the evolution of the basophil toward hyalinization. Our study does not establish conclusively the presence of this cell among the forerunners of hyaline cells.

Several investigators occasionally have found small numbers of hyalinized basophils in a variety of disease states and in normal people.[1,5,17] In the case of a patient who, after undergoing spontaneous remission of Cushing's syndrome, died of a virilizing adrenocortical carcinoma, Crooke[12] found very few hyalinized cells and claimed specificity of these cells for Cushing's syndrome. Others have reported the finding of typical hyaline basophils in cases of adrenal virilism and virilizing ovarian tumor.[9,14,16]

Corticotropin[40] and somatotropin[41] have been isolated in highly purified forms as homogeneous simple protein or polypeptide substances. Although little is known of their physical structure, analytic and physicochemical data indicate that they are different substances.[41] It is generally agreed that thyrotropin and gonadotropin fall within the group of complex glycoproteins. The recent development and application of the periodic acid-Schiff (PAS) reaction[42-45] have substantially contributed to pituitary histopathology.[15,46] Although the specificity of this reaction for glycoproteins has been accepted generally in studies of the pituitary body, Jeanloz[47] has indicated that its application in histochemistry is not completely valid. Apparently, the results of the PAS reaction are tinctorially independent of the basophilic properties of the granules.[48] The PAS reaction is thought to be based on the ability of the periodic acid to form aldehydes from glycoproteins or mucoproteins; the aldehydes in turn are made visible by the applica-

tion of leukofuchsin. All basophilic granules react positively in the normal rat and human pituitary gland. Acidophile and most chromophobe cells normally contain minute amounts of glycoprotein and are non-reactors. Since it is believed generally that chromophilic granules represent intracellular hormone, it appears reasonable to assume a similarity between basophilic granules and one or more of the glycoprotein hormones. Because there is no specific protein stain for acidophilic granules, only negative evidence correlates acidophilic granules with corticotropin and somatotropin. On the basis of experiments on rats involving endocrinopathies induced by thyroid and estrogen feeding, castration, and thyroidectomy, the specificity of these stains for the glycoprotein of basophil cells and the origin of thyrotropin and gonadotropin in the basophils have been convincingly demonstrated.[46]

Golden and associates[28] have advanced the interesting speculation that hyalinized basophils represent stored corticotropin. Later, Golden and Bondy[35] observed that Crooke's cells induced in rats by corticotropin, cortisone, and cold stress were positive reactors to the PAS reaction. Laqueur[49] suggested a corticotropin-storage phenomenon in Crooke's cells, and demonstrated that this material was PAS-positive, even in intra-adenomatous hyaline cells. Burt's[39] recent discussion of the pituitary basophils in an instance of Cushing's syndrome merits attention. While normally and sparsely granulated basophils were PAS-positive and hypertrophic amphophils were negative, significantly the hyaline basophils displayed areas of clear salmon-pink cytoplasm after the PAS reaction with an orange G counterstain, a fact which is suggestive of a positive reaction. Pearse[45] described a "virtually colorless" appearance of the hyaline material after trichrome-PAS staining. However, it is not clear whether these cells were from a case of Cushing's syndrome. In a subsequent study,[15] dealing with the Crooke cell of adrenal hyperfunction, this author indicated that PAS-positive vesicles and larger areas with an affinity for this stain were present in some of the cells, while others appeared to contain only simple protein (hyaline) material. This is in close agreement with the results of our study[50] wherein the vacuolar membrane and granular remnants were PAS-positive and the hyaline material was negative in the hyaline cells of the hormone-treated patients as well as in those with Cushing's syndrome.

These observations seem to point to the presence of a polysaccharide complex, perhaps in diminishing amounts, in the formation of the hyaline material. While the finding that hyaline material may not be PAS-positive remains consonant with the hypothesis of corticotropin

storage, no positive evidence exists to substantiate it. It would appear unlikely that the hyaline material represents stored gonadotropin, since ample evidence[27] has been offered for the presence of gonadotropic activity in women treated with cortisone, as well as in women with spontaneous Cushing's disease. Luft's studies[24] suggested normal or nearly normal gonadotropic activity in the urine in 14 cases of Cushing's syndrome of both sexes.

On the basis of the data presented it is believed that considerable evidence is at hand to support the hypothesis that Crooke's hyaline change in Cushing's syndrome is secondary to adrenocortical hyperfunction. We further conclude that the question of the cellular origin of corticotropin remains unsettled. The biochemical identification of the hyaline material could well be of importance in the study of the pathogenesis of hyperadrenocortical disorders.

Summary

The hyaline change of Crooke in the pituitary basophil cells has been acknowledged generally to be a constant pathologic finding in Cushing's syndrome. Because of the clinical and pathophysiologic similarities between Cushing's syndrome and therapeutic hypercorticoidism, the adenohypophyses of 76 patients who had received varying amounts of cortisone, corticotropin, or both substances prior to death from various unrelated diseases were studied.

The significant histologic alterations in the pituitary bodies of these hormone-treated patients were confined to the basophil cells, which, in addition to nuclear and nucleolar hypertrophy, showed varying degrees of cytoplasmic degranulation. This colloid or prehyaline change remained intensely basophilic and presented a less homogeneous appearance than the lighter staining hyaline material which ultimately formed. In general, there was a direct correlation between the degree of alteration of the basophils and the duration and amount of hormone therapy.

Although degranulated basophils represented the predominant change in the hormone-treated patients, there were also completely hyalinized basophils which were almost indistinguishable from the Crooke cell of Cushing's syndrome. It was considered that the evidence presented militated against the primary rôle of these cells in the causation of Cushing's syndrome and indicated their secondary and possibly retrogressive nature.

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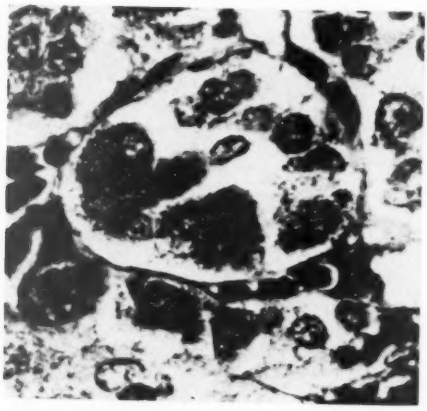
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Modified Crooke's stain of a normal pituitary body. The large cells within the central acinus are normally granulated basophils. Those just beneath are the smaller acidophils with larger granules. × 800.

FIG. 2. The granules in these basophils are small and have a finely reticulated appearance. This represents a very early cytoplasmic change. Of note are the large vacuoles. × 900.

FIG. 3. The two dark-staining cells at the left of the center are degranulated, presenting a colloid appearance. The cell above the center contains a rim of homogeneous hyaline cytoplasm and clumps of dark-staining basophilic colloid material. × 900.



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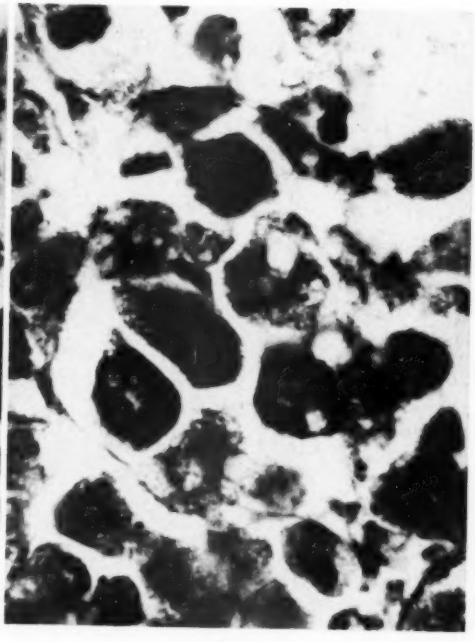

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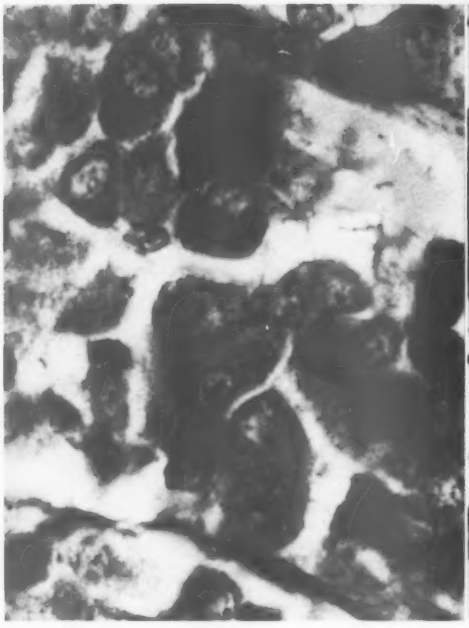

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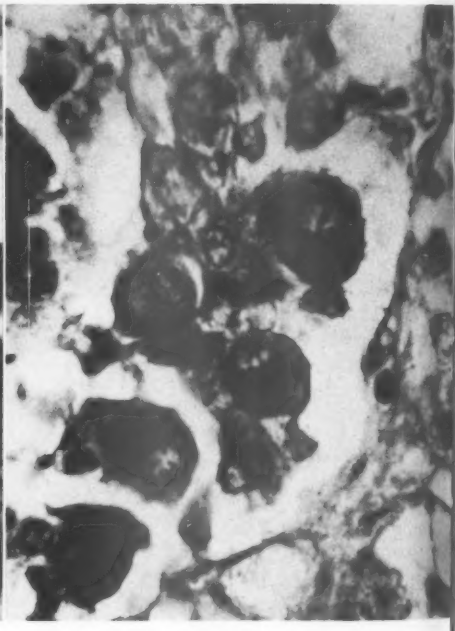

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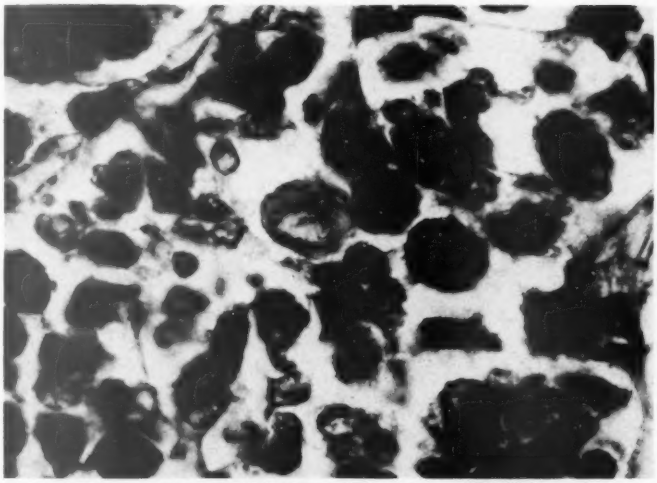
FIG. 4. Irregular strands of degranulated material have a distorting effect on the outline of these basophils. × 1,000.

FIG. 5. All basophils have a smudged, colloid appearance, easily mistaken for the hyaline change. Close examination of the cells toward the top and particularly the one at the right upper corner reveals thin rims of hyaline material. Of note are the slightly enlarged, ballooned nuclei and prominent nucleoli. × 900.

FIG. 6. The homogeneous hyaline material now occupies more of the cell volume. Residual granules and rims of dark colloid material are seen in the centrally placed cells. The cells at the lower left are in the colloid phase and contain practically no hyaline cytoplasm. × 725.



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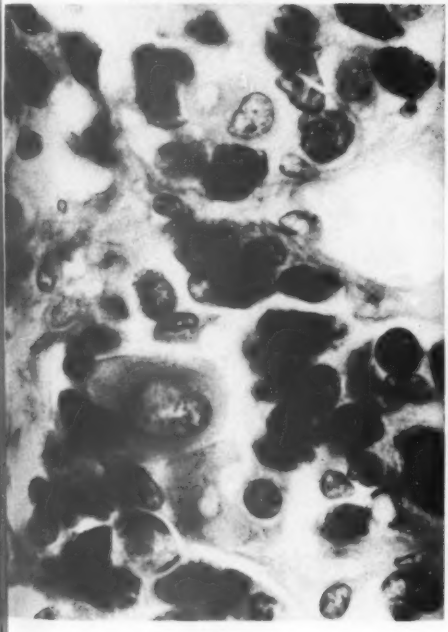

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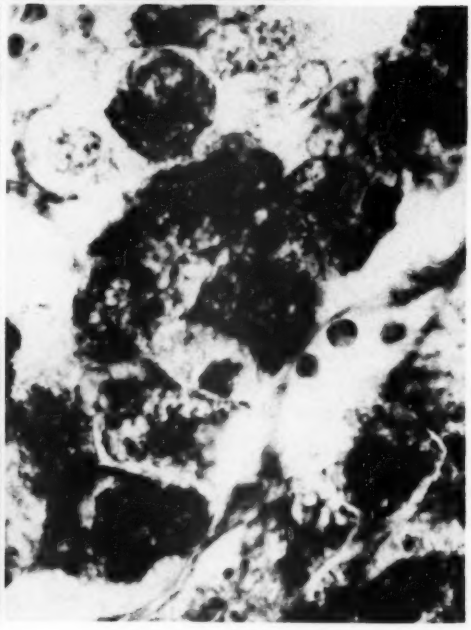

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FIG. 7. The lightly stained cell in the center is completely hyalinized, and is surrounded by degranulated, deeply basophilic cells. Except for its somewhat smaller size, this hyalinized cell is indistinguishable morphologically from the Crooke cell of Cushing's syndrome (Fig. 8). × 800.

FIG. 8. The Crooke cell of spontaneous Cushing's syndrome. The surrounding darkly staining cells approximate in size small normal basophils. × 725.

FIG. 9. Massive prolonged dosage with cortisone failed to induce hyalinization. The coarsely reticulated appearance of the cytoplasm, thought to be an early response to increased 11-oxygenated steroids, dominates the picture. × 900.

THE OCCURRENCE OF MULTIPLE FRACTURES IN SUCKLING RATS INJECTED WITH B-AMINOPROPIONITRILE (LATHYRUS FACTOR)*

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B-(γ-L-glytamyl) aminopropionitrile, a crystalline material isolated from sweet peas (*Lathyrus odoratus*) was demonstrated to be the toxic factor in the production of experimental lathyrism in rats.[1,2] It was later shown that B-aminopropionitrile (BAPN) could produce similar skeletal deformities as well as dissecting aneurysms such as those described in lathyrism.[3] Wawzonek and co-workers[4] investigated other nitrile compounds and found that amino-acetylnitrile fed to weanling rats at a level of 0.2 per cent produced severe skeletal lesions within 2 weeks. Dasler[5] investigated B-substituted ethylamines and found that B-mercapto-ethylamine was active in producing similar lesions in young rats.

The skeletal lesions of lathyrism have been described by Robinson and Bast,[6] by Lewis *et al.*,[7] and more recently by Ponseti and Baird.[3,8] Exostosis in regions of muscle attachment, osteoporosis, and kyphoscoliosis are skeletal changes that have been described. Ponseti[3] considered the alterations encountered in the skeletons of rats fed the lathyrus diet to have a clinical counterpart in several human bone disorders of unknown etiology, notably idiopathic scoliosis, adolescent kyphosis, Legg-Perthe's disease, and Paget's disease.

The histologic picture and severity of the bone lesions appear to be dependent not only upon the concentration of the toxic substance in the diet, but also on the age of the animals. Rats weighing 50 gm. develop more severe lesions within a shorter interval than do those weighing 300 gm.[3,9] Stamler[10] studied the effects of the lathyrus diet on reproduction in the rat and found poor mesenchymal development, aortic aneurysms, and scoliosis in the fetuses late in gestation as well as in the newborn. There has been no study of the effect of BAPN on suckling rats, in which bone formation is taking place at a very rapid rate. Experiments undertaken to investigate the effect of BAPN on rats from birth to 4 days of age are described.

* Supported by funds from the Atomic Energy Commission AT(11-1)-64 Project 8.
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Methods

Rat litters (Sprague-Dawley) from birth to 2 days of age were separated, and the tails of the animals selected to receive the toxic substance were snipped for identification. The suckling rats from both the control and experimental groups remained with their mothers. Each test animal was injected subcutaneously with 0.1 cc. of a 2 per cent aqueous solution of BAPN. Similar amounts were injected after 24 and 48 hours. The animals were killed at 72 hours. In other litters, larger amounts (0.3 cc.) were injected and the animals killed at 8 to 12 hours. Table I lists the number of animals and the amounts injected into individuals of the different experimental groups.

Table I

The Amounts and Times of Injection of 2 Per Cent B-aminopropionitrile

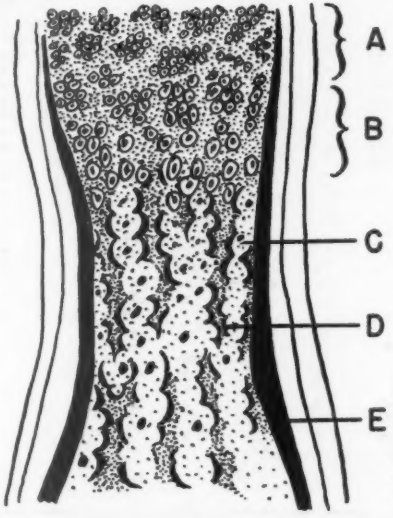
	Litter	Initial	24 Hours	48 Hours	72 Hours	96 Hours
A	L-30(6.1 gm.)	0.1 cc.	Sacrificed			
	L-31(6.2 gm.)	0.1 cc.	0.1 cc.	Sacrificed		
	L-32(6.2 gm.)	0.1 cc.	0.1 cc.	0.1 cc.	Sacrificed	
B	L-33(6.4 gm.)	0.1 cc.	0.1 cc.	0.1 cc.	Sacrificed	
	L-34(6.4 gm.)	0.1 cc.	0.1 cc.			Sacrificed
	L-35(6.1 gm.)	0.1 cc.	0.1 cc.			Sacrificed
C	L-36(6.3 gm.)	0.3 cc.	Sacrificed at 8 hours			
	L-37(6.3 gm.)	0.3 cc.	Sacrificed at 10 hours			
	L-38(6.8 gm.)	0.3 cc.	Sacrificed at 12 hours			
D	L- 8(7.0 gm.)	0.1 cc.	0.1 cc.	0.1 cc.	Sacrificed	
	L- 9(6.8 gm.)	0.1 cc.	0.1 cc.	0.1 cc.	Sacrificed	
	L-10(6.8 gm.)	0.1 cc.	0.1 cc.	0.1 cc.	Sacrificed	

A control from the same litter was removed with each injected animal.

The sacrificed animals were fixed in 10 per cent formalin after roentgenographs were made. The long bones were decalcified, embedded in paraffin, and serial sections were cut at 5 μ. Some of the sections were mounted on glass slides for staining with hematoxylin and eosin and others mounted on brass disks for historadiographic studies, a procedure that has been described previously.[11,12] Historadiography was used to determine the concentration of organic matter in five zones of bone growth as described in Text-figure 1. The zones selected were as follows: (A) central cartilage matrix, (B) matrix about hypertrophic cartilage cells, (C) central portion of the cartilage spicule, (D) edge of the cartilage spicule, and (E) perichondral bone matrix.

Results

At 48 hours, small subcutaneous hemorrhages were observed about the periphery of the long bones of the experimental animals. At 72 hours, these areas were more extensive and also became evident in the hip region and about the head of the rats. The experimental group appeared very ill at this time and each animal weighed about 8 gm. in contrast to 12 gm. for the controls from the same litter. The experiments were terminated at 72 hours since the injected animals would cease nursing or be removed from the litter by the mother.



Text-figure 1. Zones selected for historadiographic studies.

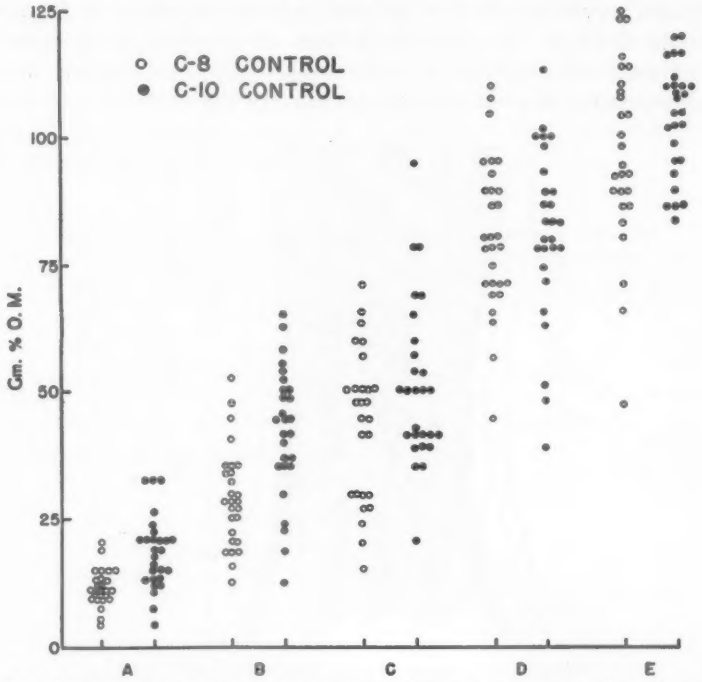
Gross x-ray examination of the injected rats (72 hours after the initial injection) revealed fractures in the shafts of the long bones and extensive osteoporosis. There was no kyphosis or scoliosis.

Dissection of the experimental animals revealed severe fractures of the long bones. Bones that were not fractured were very fragile and broke with the slightest pressure. Some of the long bones were of a rubbery consistency. On occasion, more numerous fractures were encountered, but this occurred when the mother had continued to care for the suckling rat which had struggled to nurse.

Figures 1 and 2 illustrate fractures that were observed in the experimental animals at 72 hours. In addition to fractures of this type, rents through the zone of hypertrophic cartilage cells were noted also (Fig. 3). Ponseti[3] also described rents in older animals that were fed a diet

containing sweet peas. These rents may be the site of epiphyseal fractures, as is illustrated in Figure 4.

Histologic studies of the long bones from treated rats revealed moderate degeneration of the osteoblasts at 24 hours. At 48 hours, the change from basophilia to a granular eosinophilia was pronounced in

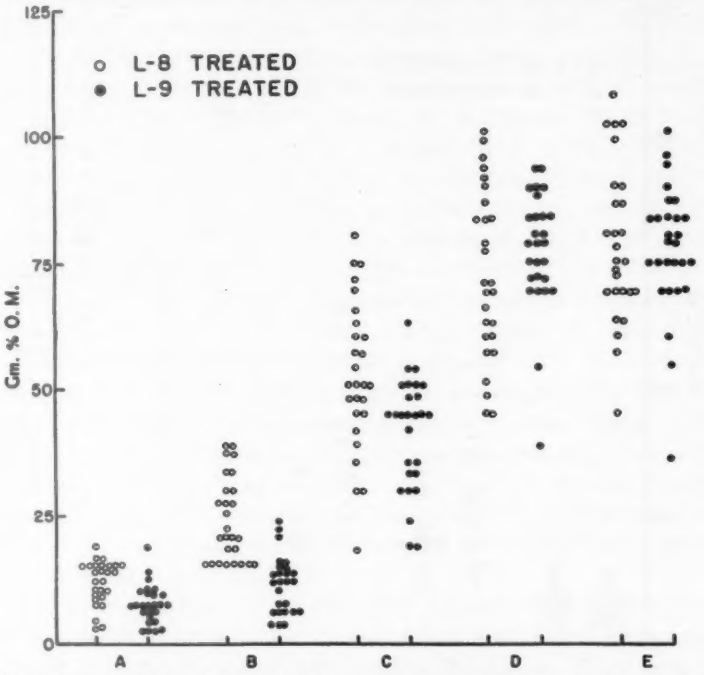


Text-figure 2. Historadiographic data illustrating varying concentration of organic matter in different zones of the femur. C-8 and C-10 are controls for litter D. (See Text-fig. 1.)

most of the osteoblasts. Many of these cells were enlarged, spherical, and extremely vacuolated. There appeared to be a fusion of these degenerating cells, giving rise to cells possessing many of the characteristics of osteoclasts (Fig. 5). At 72 hours, few normal osteoblasts or large vacuolated cells were evident. All that remained were osteoblasts with a small amount of an eosinophilic cytoplasm in addition to large numbers of immature osteoblasts or fibroblasts. These cells were capable of forming a fibrous matrix at sites of callus formation (Fig. 2). Apparently there was no interference with the ability of mesenchymal cells to differentiate into cartilage cells, since these cells were present in the callus.

Fractures were present also in animals given only two injections of the nitrile and sacrificed at 96 hours (Table I). In this group there was more callus formation and extensive proliferation of fibroblasts in portions of the marrow cavity.

The degenerative changes in the osteoblasts were greatly accentuated



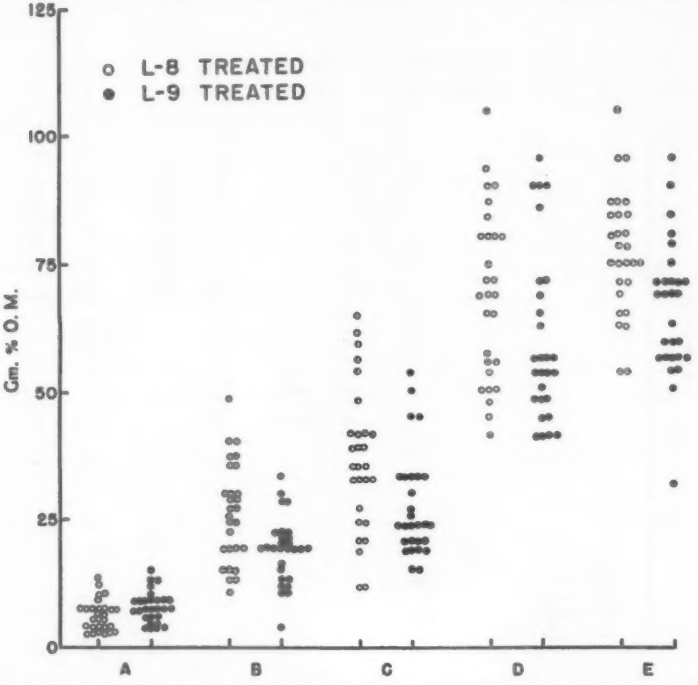
Text-figure 3. Historadiographic data illustrating varying concentration of organic matter in different zones of the humerus. L-8 and L-9 are from litter D.

in animals given a single injection (0.3 cc. of the 2 per cent solution) of BAPN. Eight to 12 hours following the injection of this amount, all of the osteoblasts were either spherical, with eosinophilic cytoplasm, or extremely vacuolated and had coalesced to form multinucleated cells resembling "osteoclasts" (Fig. 6). No fractures had occurred in these animals.

Historadiographic studies enabled an *in situ* determination of the concentration of organic matter in different areas of the bone. The data from these measurements are presented graphically in Text-figures 2 (control), 3 (treated, humerus), and 4 (treated, femur).

In the control animals (Text-fig. 2) there is a variation in the concentration of organic matter at different areas. The concentration is

lowest in the matrix about immature cartilage cells (zone A), but increases in amount about hypertrophic cartilage cells and is at the highest value in the matrix of endochondral and periosteal bone (zone E). Previous studies[13] revealed that these changes in concentration parallel the deposition of bone salts and appear essential for normal



Text-figure 4. Historadiographic data illustrating varying concentration of organic matter in different zones of the femur. L-8 and L-9 are from group D.

calcification of the cartilage or bone matrix. In the experimental groups, the organic matter of the matrix is decreased in concentration in all zones. This decrease in organic matter is apparently associated with the degree of osteoporosis and also with the degenerative changes in the osteoblasts.

The matrix in the region of rents also was studied by the historadiographic procedure. Data from the control animals (Text-fig. 2) indicate that as cartilage cells hypertrophy, the surrounding matrix increases in density because of a concentration of organic matter. If this increased concentration did not occur, the area would become a zone of structural weakness. The concentration of organic matter in the zone of hypertrophic cartilage cells (zone B) adjacent to a rent was

determined (L-9 in Text-fig. 3). In this case an increase in the concentration of organic matter had not taken place in the hypertrophic region.

Discussion

The normal development and function of the osteoblast in the formation and maintenance of bone matrix is not well understood. Osteoblasts appear to develop from mesenchymal cells which enlarge and accumulate large amounts of ribonucleic acid in their cytoplasm. This accumulation of ribonucleic acid has been associated with the possible rôle of these cells in the formation of the protein matrix of bone.[14] Arey[15] has described a progressive degeneration of the osteoblasts which he considers the change leading to bone dissolution. A study of the histologic alterations occurring in the bones of the control group and animals injected with BAPN adds support to Arey's observations and offers a possible explanation for the occurrence of the fractures.

Among the control animals, degenerative changes in the osteoblasts can be detected and there is as well a larger number of "osteoclasts" than are observed in older animals. It appears that the osteoblast undergoes a normal pattern of development and degeneration that may be associated with simultaneous changes in the protein matrix. Osteoblasts containing a deep basophilic cytoplasm are possibly more active in forming and maintaining the bone matrix than are degenerating cells characterized by an eosinophilic and/or vacuolated cytoplasm. Dissolution of the matrix may be associated with cells of the latter type in addition to the "osteoclast."

This normal pattern for the osteoblast appears to be accelerated by BAPN since one can observe in the injected animals a pronounced increase in the incidence of degenerating osteoblasts. These cells enlarge and fuse to form multinucleated cells that resemble "osteoclasts." A more careful quantitative study of the chemical changes occurring in osteoblasts and bone matrix is required before definite statements can be made about the rôle of the osteoblast and its relationship to changes that take place in the bone matrix. The occurrence of multiple fractures resulting from the administration of BAPN is centered about the relationship of the osteoblast to the bone matrix. An attempt to solve this problem was made by comparing the historadiographic readings on comparable bones from control and experimental animals. The results show that an increased concentration of organic matter is present at sites of greater calcification. The injection of BAPN produces severe osteoporosis and subsequent fractures in young rats. The osteoporosis is associated with a large decrease in the concentration of organic

matter in the bone matrix and also with pronounced degeneration of the osteoblasts. Immature osteoblasts or fibroblasts may proliferate but fail to produce normal bone. Callus formation is not interfered with; however, the matrix in this callus does not calcify.

In some manner the lathyrus factor initiates or accelerates a degenerative process occurring in cells that build bone or cartilage matrix. The result is an inability of the matrix to calcify or the lack of maintenance of an already formed matrix. These factors lead to the occurrence of rents and other points of structural weakness in the cartilage or bone, with subsequent fracture.

The multiple fractures produced and their histologic appearance are suggestive of osteogenesis imperfecta. More studies will be necessary to establish whether there is any relationship.

Summary

Multiple fractures were produced by injecting B-aminopropionitrile (lathyrus factor) into suckling rats. These fractures were preceded by pronounced degeneration of the osteoblasts. Quantitative historadiographic studies indicated that there is a dissolution of the organic matrix associated with osteoporosis and the degenerative changes seen in the osteoblasts. There was no interference with the ability to form callus, although the matrix of the callus was abnormal. There is a conspicuous similarity between the lesions produced by this chemical and the bone lesions found in osteogenesis imperfecta.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Fracture of the humerus from a treated animal from litter D. × 70.

FIG. 2. Fracture of tibia and fibula. Animal from litter D to show callus. × 70.

FIG. 3. Rent in zone of hypertrophic cartilage cells of the humerus. Animal from litter D. × 190.

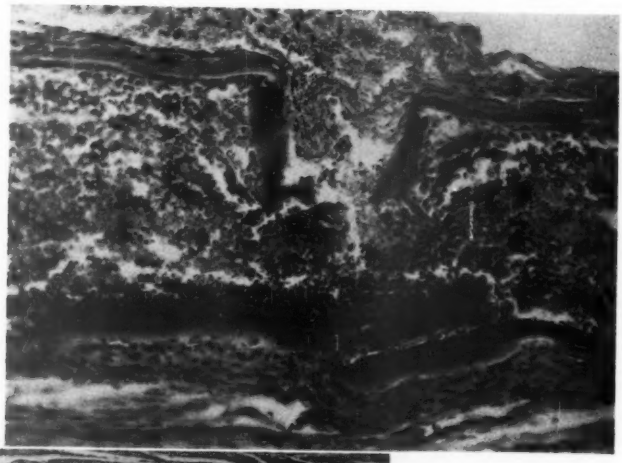

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FIG. 4. Separation and displacement of femoral epiphysis. Animal from litter D. × 30.

FIG. 5. Degenerating osteoblasts. Animal from litter A, L-31. × 860.

FIG. 6. Multinucleated cells resembling "osteoclasts." Litter C. × 690.

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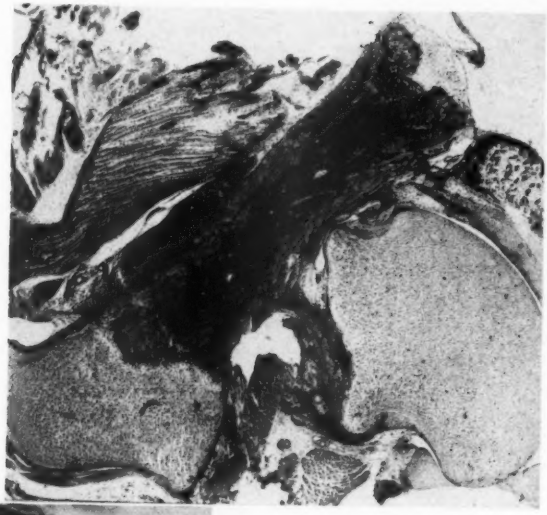

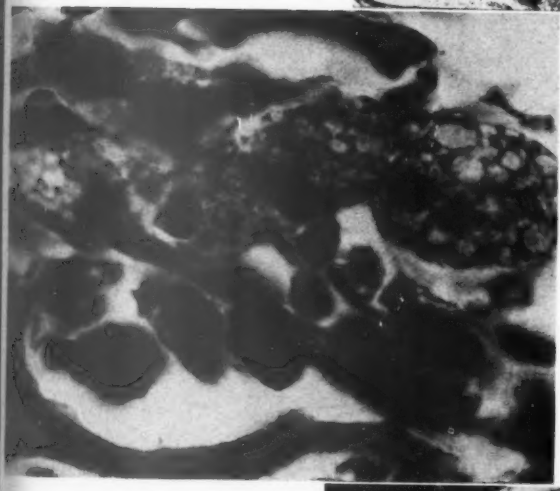

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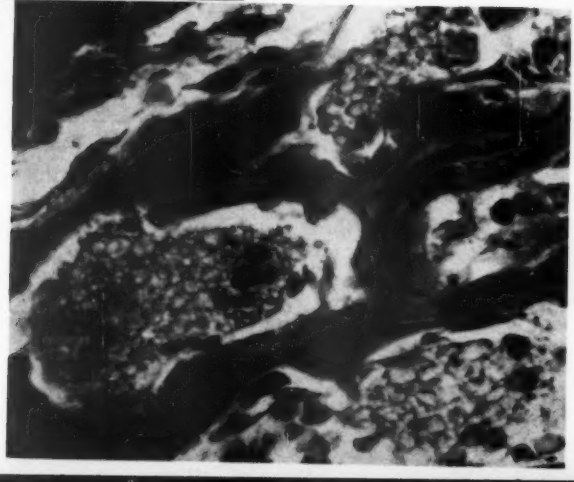

DISCRETE SPLENIC CALCIFICATION IN NECROPSY MATERIAL*

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Splenic calcification may be produced by numerous pathologic processes, and the exact pathogenesis is often difficult to establish. The most common type shows multiple small discrete foci scattered throughout the splenic parenchyma. These usually measure less than 1 cm. in diameter and may be present as single foci or, more commonly, in groups. This study is a survey of the spleens from 100 consecutive necropsies in an effort to determine the incidence and causation of these discrete foci of calcification in this geographic area.

The earlier investigators believed that such foci of calcification were phleboliths, but more recent reports lean toward interpreting them as the end results of focal granulomatous lesions. Tuberculosis has been proved a common cause for such lesions. Reichle and Work,[1] in 1939, found focal calcification in 73 of 500 necropsies and inoculated guinea-pigs with material from 14 cases. Of these, 3 cases gave positive results. In 1912 it was stated by Winternitz[2] that two thirds of the children and 20 per cent of the adults dying of tuberculosis had splenic involvement. Gray,[3] in 1944, found calcification in 63 of 111 spleens, and 8 were from patients with active pulmonary tuberculosis. Many earlier surveys dealt with splenic roentgenographic findings only.

More recently High,[4] in surveying x-ray films of the chests of school children in Kansas City, Missouri, found 20 with splenic calcification, and 84 per cent of these revealed positive histoplasmin skin tests. Only 47 per cent gave positive tuberculin skin tests. The possible relationship of histoplasmosis to splenic calcification was suggested.

Schwarz *et al.*,[5] in 1955, surveyed spleens from surgical cases and from necropsies at Rotterdam, New York City, and Cincinnati. The figures for incidence of calcification were 29, 27, and 62 per cent, respectively. For only one case, however, were cultures done. The Gridley stain for fungi demonstrated forms interpreted as *Histoplasma capsulatum* in 19 cases, representing 47 per cent of those showing "typical" lesions. The authors believed that it often was possible to distinguish between lesions produced by histoplasmosis and those of tuberculosis, the former being round and discrete.

* Received for publication, April 23, 1956.

Method

In 100 consecutive necropsies the spleens were sectioned with maintenance of a sterile technique. Foci of calcification were removed, ground up, smeared, and cultured for tubercle bacilli and fungus organisms. The media used for culturing tubercle bacilli were Petragnani's and that of the American Trudeau Society incubated at 37° C. For fungi, Thompson's medium was incubated at 37° C. and Sabouraud's medium at 25° C. The Thompson medium was dispensed in tubes which were plugged with corks and sealed with paraffin to conserve moisture. Cultures for tubercle bacilli were observed for 8 weeks, and those for fungus organisms for 5 weeks. If more than one focus of calcification was present, sections were prepared for microscopic study by hematoxylin and eosin, acid-fast, periodic acid Schiff (PAS), and Gridley's stains.

Results

In the 100 cases there were 46 with splenic calcification. Fourteen of these had single nodules and 32 had multiple foci, ranging to almost 100 in number and up to 1 cm. in diameter (Fig. 1). Of the 46 positive cases, 31 patients were white and 15 Negro. This is approximately the same ratio as exists in admissions to this hospital and that existed in the necropsy series of 100 cases. One patient died with culturally proved active pulmonary histoplasmosis and the spleen revealed one focus of calcification. Three patients died with proved active pulmonary tuberculosis and all had calcification in the spleen. In one other case active granulomatous lesions were demonstrated in the lungs and calcification in the spleen. A 26-year-old Negro man died as a result of quadriplegia following an automobile accident and the lungs and spleen revealed active granulomatous lesions. Most of the patients with splenic calcification had lived in Tennessee, Mississippi, Arkansas, or Missouri. One each had lived in Ohio, Michigan, Kentucky, Louisiana, Illinois, and Alabama.

In not a single instance did the smears or cultures for acid-fast or fungus organisms disclose these agents. In only one of the 32 cases in which histologic sections were prepared were forms revealed suggestive of Histoplasma organisms, and these occurred near the fibrous capsule. No organisms were found in the centers of the caseous material in any of the slides (Fig. 4). All acid-fast stains were negative.

The histologic appearance of the lesions was similar to that described by many others. A capsule of dense hyaline fibrous tissue of varying thickness surrounded a central zone of caseous or granular material in which varying degrees of calcification were present (Fig. 2). Occa-

sional cholesterol deposits were encountered. The necrotic débris in the center varied in its affinity for the PAS stain. The foci stained poorly or not at all by the Gridley technique. Often the central débris near the capsule contained globules of lipid-like material which varied greatly in size and usually did not occur in clusters (Fig. 3). The shells of these globules stained varying shades of blue with hematoxylin and eosin. They often had a PAS-positive shell but no identifiable features which would allow them to be interpreted as *H. capsulatum*. These forms were usually stained only faintly with the Gridley technique. It is our interpretation that these droplets are products of tissue breakdown. Often near and in the fibrous capsule of the calcific foci there were fragmentation of tissue and formation of small fragments and droplets which frequently stained positively by the PAS and Gridley methods. These fragments can, when stained positively, make difficult the finding of Histoplasma organisms. Control slides, of course, were used for the staining procedures.

Discussion

In the past 10 years, special staining techniques for the demonstration of many substances in tissue sections have become popular and very useful. In 1946, McManus[6] reported the use of periodic acid oxidation followed by treatment with Schiff's fuchsin-sulfurous acid for staining of mucin. Since then the PAS staining method has been used to demonstrate many tissue substances[7] and fungi. Its use for the identification of fungi in tissue sections began with the work of Kligman *et al.*[8,9] The PAS method often stains much other particulate material in sections, and this has detracted from its value as a screening method for identification of fungi. Because of this, Gridley[10] developed a similar stain, using chromic acid as the oxidant instead of the stronger periodic acid. This supposedly eliminated the positive staining of tissue elements. Our experience with these two methods shows the Gridley stain to be much better as a screening method for fungi, but some tissue elements do stain positively. This has been the experience of others[11] also. Kade and Kaplan[11] recommended the Gridley and the Brown-Brenn stains for identification of Histoplasma organisms in tissue sections.

Puckett, in 1953[12] and 1955,[13] was able to identify forms interpreted as *H. capsulatum* in all 67 cases in which localized pulmonary lesions had been resected. He used the PAS stain. Cultures for fungi and acid-fast organisms were negative in all but one case in which histoplasmosis was proved culturally.

Histoplasma organisms may present somewhat unusual forms in tissue sections. A consideration of the large and small forms is given by Weed[14] and Schwarz and Straub.[15,16] Earlier, the organisms were usually identified only intracellularly, but the more recent work has shown numerous examples of extracellular forms. Binford[17] and Zimmerman[18] also stressed the morphologic variations of this fungus in tissue. Puckett[12] believed these forms were non-viable since they were not recovered by culture or animal inoculation. This makes the definitive diagnosis of histoplasmosis quite difficult. Most of these investigators have noted the artifacts produced by PAS and Gridley staining methods which frequently may be confused with various fungus organisms. Starr, Dawe, and Weed[19] pointed out that these morphologic methods do not supplant cultural methods and may not be a valid basis for positive identification of a disease such as histoplasmosis. This certainly has been our experience and is our belief.

Most of the cases in our survey came from the area described as the endemic region for histoplasmosis in the United States.[20,21] In all probability the incidence of splenic calcification varies in different regions and results from a variety of causes. Our incidence of 46 per cent is relatively high and compares with that of Schwarz.[5] We were able to find only one case, however, in which structures were found which could be interpreted as probable organisms of histoplasmosis. In the disseminated form of histoplasmosis the adrenal gland is frequently involved,[22] but in our series not a single case revealed calcification or active granulomatous inflammation of an adrenal gland. The material comprising a part of Schwarz's[5] series was drawn from Cincinnati, which is more nearly in the central part of the endemic region, while our material is nearer the fringe. We firmly believe that further investigation with correlation of data from skin testing, complement-fixation, clinical studies, and animal inoculation are necessary before one assumes that histoplasmosis is the common cause of splenic calcifications.

One of our cases and another in Schwarz's[5] series presented interesting findings. Active focal granulomatous lesions were present, but smears and cultures failed to reveal the causative organism. We have seen one other case in which the spleen was removed surgically and contained large and small granulomatous lesions which failed to show organisms on smear, culture, and special stains. This suggests that in some of these splenic lesions the organisms are destroyed completely quite early, or that the lesions may be the result of a tissue immunity such as suggested by Pinkerton and Iverson,[23] though here the lesions show necrosis of a caseating type.

While *H. capsulatum* may at times be difficult to grow, we consider the cultural isolation or presence of completely typical organisms mandatory for a definitive diagnosis.

Summary

A study of the spleens from 100 consecutive necropsies revealed small foci of calcification in 46. In none could acid-fast or fungus organisms be found on smear or culture. In one case, forms consistent with variants of *Histoplasma capsulatum* were found in microscopic sections. A conservative interpretation of positive staining material in the PAS and Gridley methods seems indicated.

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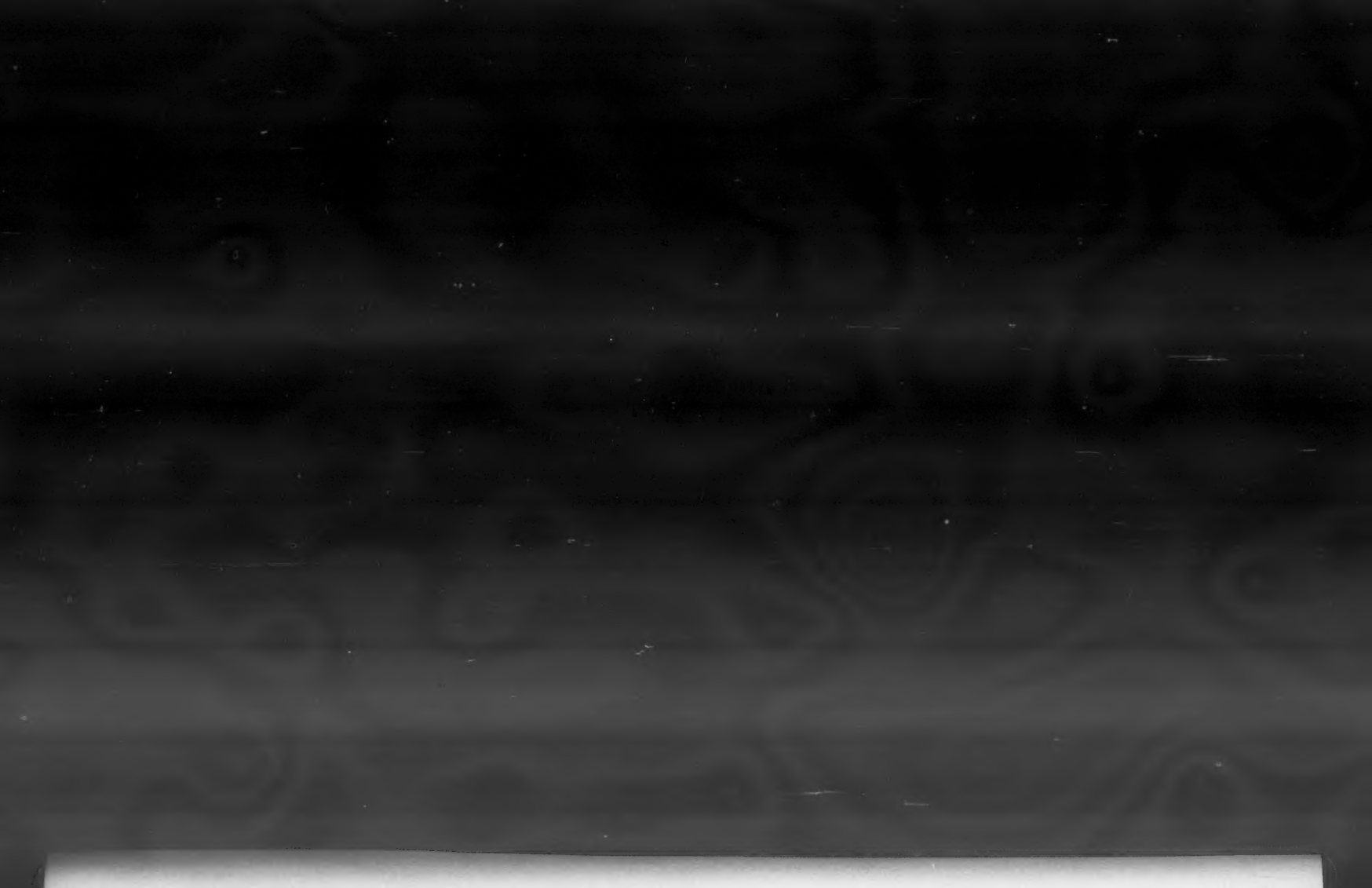
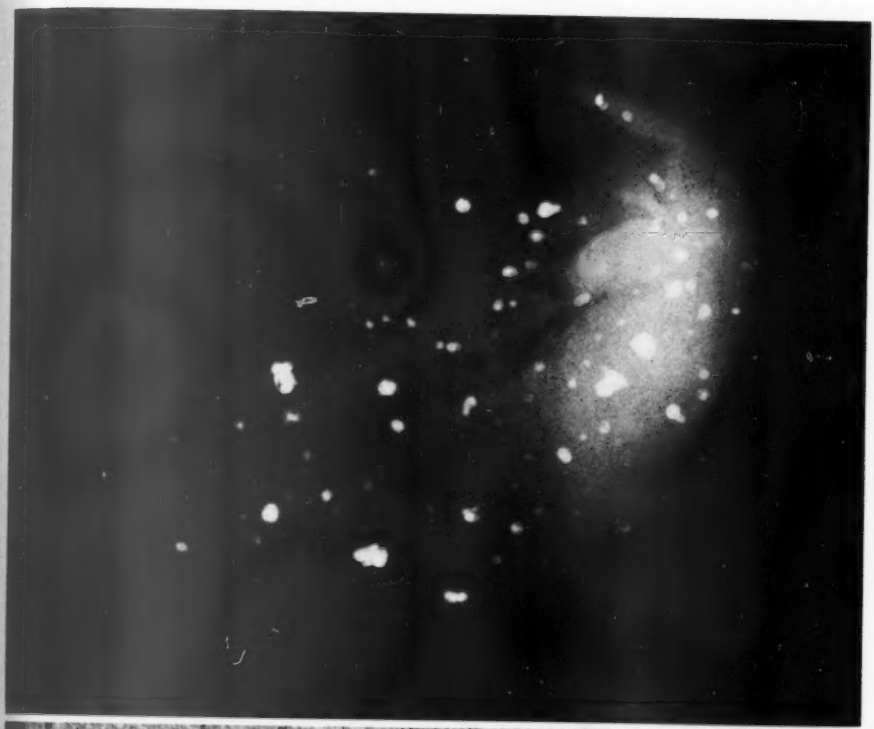
LEGENDS FOR FIGURES

FIG. 1. Radiographic appearance of spleen with multiple discrete foci of calcification.

FIG. 2. Low-power magnification of decalcified splenic lesion, showing the discrete margin and the solid structure of the lesion.





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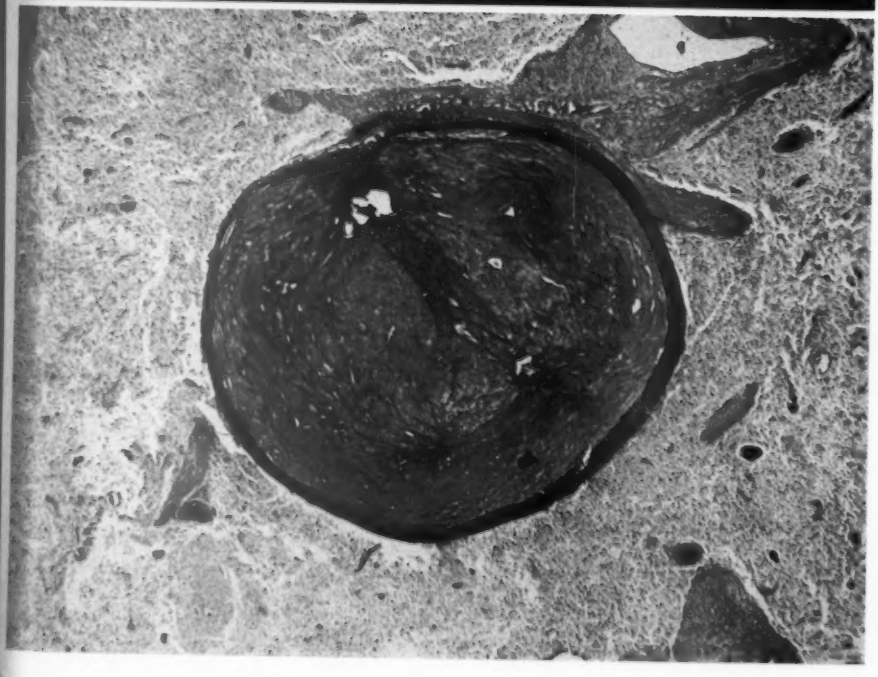
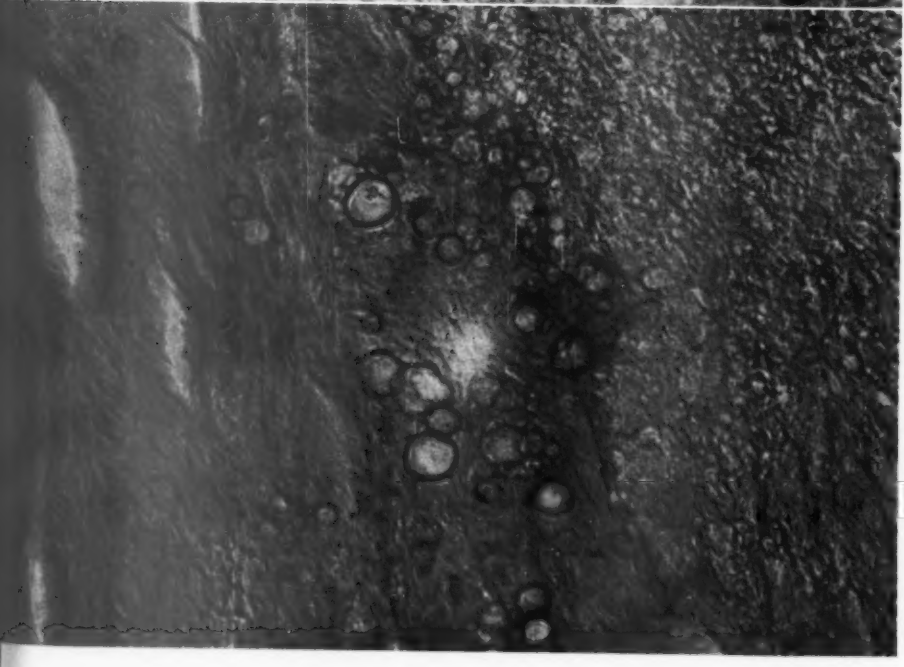
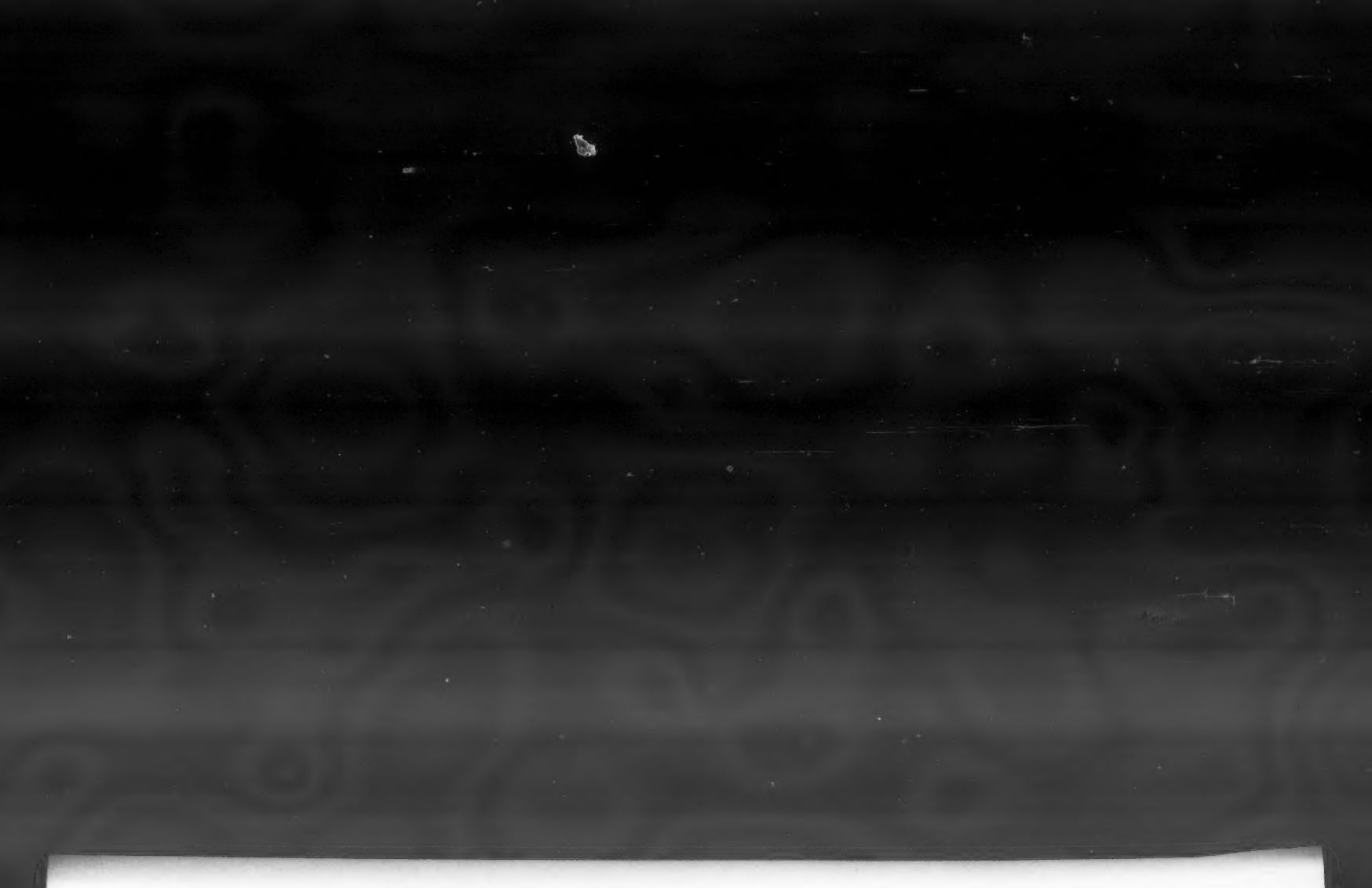


2

FIG. 3. This section reveals clusters of ring forms similar to those now called atypical *Histoplasma capsulatum* organisms. Gridley stain. × 1,000.

FIG. 4. Amorphous particulate material often found near margins of splenic lesions. Hematoxylin and eosin stain. × 1,000.

3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